The role of 14q32 microRNAs in vascular remodelling

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Chapter 7

Posttranscriptional regulation of 14q32 microRNA miR-329 during vascular regeneration after ischemia

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Abstract

The human imprinted 14q32 locus holds the largest known microRNA gene cluster, encoding 54 individual microRNAs. We have previously shown that the 14q32 microRNA cluster plays a role in neovascularization. After induction of ischemia in mice, 14q32 microRNAs are regulated in three distinct temporal patterns. These expression patterns, as well as basal expression levels, were independent of the microRNA genes' order in the 14q32 locus. These findings indicate that posttranscriptional processing of 14q32 microRNA precursors is a major determinant of 14q32 microRNA expression.

Using rt/qPCR primers specific for each stage of microRNA processing, i.e. pri-microRNA, pre-microRNA and mature microRNA, we found that increased expression of 14q32 microRNAs after hindlimb ischemia is determined by increased processing from pre- to mature microRNA, rather than increased transcription. We used Stable Isotope Labelling of Amino Acids (SILAC), followed by pre-microRNA pull-down and Mass Spectrometry to identify proteins that are responsible for posttranscriptional regulation of 14q32 microRNA precursors.
We observed differential binding of proteins CIRBP and HADHB to the precursors of late-responder miR-329-3p and non-responder miR-495-3p. Immunohistochemical staining confirmed expression of both CIRBP and HADHB in the adductor muscle of mice and expression of both CIRBP and HADHB was upregulated after hind limb ischemia. Using RNA Binding Protein immunoprecipitation (RIP) experiments, we showed specific binding of CIRBP to pre-miR-329, but not to pri-miR-329. Finally, inhibition of CIRBP, using RNAi, reduced miR-329-3p and miR-495-3p levels in 3T3 cells.

These data demonstrate a novel role for CIRBP and HADHB in posttranscriptional regulation of 14q32 microRNAs under ischemia.

Introduction

MicroRNAs are short endogenous RNA molecules (~22 nucleotides) that decrease expression of their target genes via translational repression[1]. MicroRNA genes are transcribed by RNA polymerase II as primary microRNA (pri-miR) transcripts. Subsequently, these pri-miRs are processed by the microprocessor complex, consisting of the RNase III Drosha and co-factor DGCR8, to form precursor microRNAs (pre-miRs) of about 70 nucleotides long. Pre-miRs are exported to the cytoplasm where the enzyme Dicer cleaves the pre-miR into a microRNA duplex. Generally, one strand of the microRNA duplex (guide strand) is preferred for association with an Argonaute (AGO) protein and loading into the RNA induced silencing complex (RISC). However, accumulating evidence suggests that the other strand (passenger strand) can also be loaded into the RISC[2, 3]. MicroRNAs guide the RISC to specific mRNA targets, in order to control mRNA translation[1]. A single microRNA is able to target numerous genes and by doing so, that microRNA can regulate complex, physiological processes. Over the past decade, microRNAs have been shown to play an important role in human disease, including cardiovascular disease. Although microRNAs regulate physiological and pathological processes via modulation of target gene expression, microRNA expression itself is subject to regulation too.

MicroRNA expression can be regulated at transcriptional as well as posttranscriptional level. Processing of microRNA precursors can be controlled at the conversion of pri-miR to pre-miR through modulation of Drosha/DGCR8 activity and at the conversion of pre-miR to mature microRNA through modulation of Dicer activity. RNA binding proteins (RBPs) have been found to bind sequences in the terminal loop and stem of pri-miRs, thereby enhancing or inhibiting pri- to pre-miR cleavage[4-7]. For example, processing of pri-miRs with conserved terminal loop regions, such as pri-miR-18a or pri-let-7a have been shown to be affected by hnRNP A1 protein[7-9]. Furthermore, a subset of murine microRNAs was affected by depletion of p68 and p72 helicases, resulting in reduced levels of pre-miRs, but not pri-miRs[10]. In addition, p53 and SMADs have been reported to (in)directly interact with Drosha and modulate pri- to pre-miR cleavage[6, 11]. The RNA binding protein LIN-28 was reported to block accumulation of let-7 levels by repression of both Drosha and Dicer[12]. Another RBP, AUF1, was shown to interact with Dicer and lowered Dicer mRNA stability, leading to a decrease in mature microRNA levels, but not pre-miR levels[13]. Recently, Rbfox proteins were found to bind pre-miRs of miR-20b and miR-107 through specific Rbfox RNA recognition motifs in their terminal loops and Rbfox2 was shown to decrease mature miR-20b and miR-107 levels[14].

Many microRNAs are encoded within clusters and can be transcribed as long polycistronic transcripts. With 54 microRNA precursors, the 14q32 cluster is the largest known polycistronic microRNA gene cluster in humans. In mice, this cluster is located on chromosome 12F1 and contains 61 microRNAs. We have previously described the differential regulation of 14q32 microRNAs in a mouse model for ischemia in the hind limb[15]. MicroRNAs from the 14q32 cluster followed three different expression patterns after induction of ischemia. These patterns are independent of the chromosomal location of the 14q32 microRNA genes. Furthermore, even baseline expression levels of these 14q32 microRNAs vary greatly. These findings indicate that individual 14q32 microRNA expression is regulated predominantly at posttranscriptional level.

In this study, we show that 14q32 microRNAs are indeed regulated at posttranscriptional level. We identified RBPs that bind and process specific 14q32 microRNA precursors. This helps to explain the differential expression of 14q32 microRNAs under ischemia and expands our knowledge of regulation of microRNA biogenesis under pathological conditions.

Methods

Hind limb ischemia model

All experiments were approved by the committee on animal welfare of the Leiden University Medical Center (Leiden, the Netherlands. Approval reference numbers 09163 and 10243). This study was conducted in accordance with the Dutch government guidelines and the Directive 2010/63/EU of the European Parliament. Unilateral hind limb ischemia was induced in healthy adult male C57BL6 mice by single ligation of the left femoral artery, as previously described[15]. Briefly, electrocoagulation of the femoral artery was performed proximal to the superficial epigastric artery (single ligation model) or combined with electrocoagulation of the distal femoral artery, proximal to the bifurcation of the popliteal and saphenous artery (double ligation model)[16]. C57Bl/6 mice (n=4 per timepoint) were sacrificed at several timepoints (day 0 (before ligation of the femoral artery), day 1, 3, 7, 10, 14 and 28) after hind limb ischemia induction. Upon sacrifice, the adductor and gastrocnemius muscles were harvested and either snap-frozen on dry ice or fixed in 4% paraformaldehyde.

Microarray

For microarray analysis, total RNA was isolated from adductor muscles using the RNeasy fibrous tissue minikit (Qiagen). RNA concentration, purity and integrity were analysed by nanodrop (Nanodrop® Technologies) and Bioanalyzer (Agilent 2000) measurements. Animals

For microRNA expression profiling, adductor muscle tissue of day 0, 1, 3 and 7 after induction of hind limb ischemia was used. MicroRNA expression profiling was performed as previously described, using LNA based arrays (miRCURY LNA™ miR Array ready-to-spot probe set, Exiqon)[15]. Normalization and background correction was performed in the “statistical language R” using “vsn” package (Bioconductor). Differential expression was assayed using the “limma” package (Bioconductor) by fitting the eBayes linear model and contrasting individual treatments with untreated controls. Log2 fold changes were calculated using the toptable function of the limma package.

For whole genome expression profiling, adductor muscle tissue of day 0, 1, 3, 7, 14 and 28 after induction of hind limb ischemia was used. Whole genome expression profiling was performed using MouseWG-6 v2.0 Expression Beadchips (Illumina) and expression levels were Log2-transformed, as previously described[15].

Cell culture

3T3 cells were cultured at 37ºC in a humidified 5% CO_2 environment. Culture medium consisted of DMEM GlutaMAX™ (Gibco) supplemented with 10% heat inactivated fetal calf serum (PAA) and 1% penicillin/streptomycin (PAA). Culture medium was refreshed every 2-3 days. Cells were passed using trypsin-EDTA (Sigma) at 90% confluency.

In vitro regulation of microRNA expression

To mimic the differential microRNA expression after *in vivo* ischemia, 3T3 cells were cultured under serum starvation conditions (DMEM glutaMAX™ supplemented with 0.5% heat inactivated fetal calf serum and 1% penicillin/streptomycin). Cells were starved overnight under serum starve conditions (~16 hours) and the next morning cells were either placed on starve medium (0.5% FCSi) or stimulation medium (10% FCSi) for 24 or 48 hours.

RNA pull-down and SILAC Mass Spectrometry

RNA pull-down and mass spectrometry were performed as described previously, with slight modifications[17]. In brief, total protein extracts from normal serum and serum starved 3T3 cells grown in ‘light’ [^{12}C]Arg/[12C]Lys and ‘heavy’ [^{13}C]Arg/[^{13}C]Lys isotopes, respectively, were incubated with in vitro transcribed RNAs chemically coupled to agarose beads. The incubation was followed by a series of washes with buffer G (20 mM Tris pH 7.5, 135 mM NaCl, 1.5 mM MgCl2, 10% (v/v) glycerol, 1 mM EDTA, 1 mM DTT and 0.2 mM PMSF). After the final wash, the proteins associated with the RNA on the beads were analyzed by SDS–PAGE followed by in-gel digestion and mass spectrometry or western blotting.

Western Blot Analysis

Total protein samples from 3T3 cells (100 µg per lane), isolated by sonication, were resolved by standard NuPAGE SDS–PAGE electrophoresis with MOPS running buffer (Life Technologies) and transferred onto a nitrocellulose membrane.

Total protein samples (5 µg per lane) from murine adductor muscle tissue were isolated using a standard TRIzol protocol (Thermo Fisher) at day 0, 1, 3 and 10 after induction of ischemia (double ligation model). The membrane was blocked overnight at 4°C with 1:10 western blocking reagent (Roche) in TBS buffer with 0.1% Tween-20 (TBST). The following day, the membrane was incubated for 1 h at room temperature with primary antibody solution in 1:20 western blocking reagent diluted in TBST in the following concentrations; rabbit polyclonal CIRBP (Protein Tech 10209-2-AP) 1:1000, rabbit polyclonal HADHB (LSBio-LS-C334236) 1:500. After washing in TBST, the blots were incubated with the appropriate secondary antibody conjugated to horseradish peroxidase and detected with SuperSignal West Pico detection reagent (Thermo Scientific). The membranes were stripped using ReBlot Plus Strong Antibody Stripping Solution (Chemicon) equilibrated in water, blocked in 1:10 western blocking solution in TBST and re-probed as described above.

Western Blots were quantified using ImageJ analysis software (1.48v, NIH) and normalized to the input.

RNA Binding Protein Immunoprecipitation

RNA Binding Protein immunoprecipitation (RIP) was performed using the EZMagna RIP kit (Millipore), according to manufacturer's instructions. 3T3 cells were grown to 90% confluency and lysed in complete RIP lysis buffer. Cell lysates were incubated with RIP buffer containing magnetic beads conjugated with antibodies against Cold Induced RNA binding protein (CIRBP, Abcam ab106230), hydroxyacyl-CoA dehydrogenase/3-ketoacyl-CoA thiolase/enoyl-CoA hydratase (trifunctional protein), beta subunit (HADHB, Novus Biologicals NBP1-82609), SND1 (Abcam ab71186) and rabbit control IgG (Millipore PP64B). Before immunoprecipition, 10% of cell lysate was taken and served as input control. Next, samples were treated with proteinase K to digest protein and RNA was isolated using a standard TRIzol-chloroform extraction protocol.

RNA interference

The following sense siRNA sequences (Sigma-Aldrich) were used to target CIRBP in 3T3 cells;

CIRBP1: GAGACAGCUAUGACAGUUAUU and *CIRBP2: GUGGUAAAGGACAGGGAGA.*

SiRNAs were transfected using Lipofectamine 2000 (Life Technologies) in either a single event or two events separated by 48h, as indicated. A concentration of RNA in Opti-MEM medium of 400nM was ensured, following manufacturer's instructions.

RT/qPCR

Adductor and gastrocnemius muscles from day 0 and day 1, 3 and 7 after surgery were homogenized by grounding with a pestle and mortar in liquid nitrogen. Total RNA was isolated using a standard TRIzol-chloroform extraction protocol. RNA concentration and purity were determined by nanodrop (Nanodrop® Technologies). RNA was reverse transcribed using high-capacity RNA to cDNA RT kits (Life Technologies, USA). Relative quantitative mRNA PCR was performed on reverse transcribed cDNA using, SYBR® green dye (Qiagen). Primers for pri-miRs, pre-miRs, HADHB and CIRBP were designed using Primer3. Sequences of primers are listed in Supplementary Table 1. MicroRNA quantification was performed using Taqman® microRNA assays (Applied Biosystems) according to manufacturer's protocol. Relative quantitative PCR was performed on the Viia7 system (Applied Biosystems) and amplification efficiencies were checked by standard curves. Data were normalized using a stably expressed endogenous control (snRNA-U6).

Levels of mature microRNAs in serum-starvation experiments on 3T3 cells were measured using miRScript RT (Qiagen) and SYBR® green (Qiagen). MicroRNA levels were normalized to microRNA-16.

Immunohistochemical staining

Formaldehyde fixed adductor muscles were paraffin-embedded and 5 µm thick cross-sections of muscles were stained to visualize expression of RNA binding proteins. Cross sections of adductor muscles were re-hydrated and endogenous peroxidase activity was blocked. Antigen retrieval was performed with Citrate buffer (pH 6.0) at 100ºC for 10 minutes. Muscles were stained with rabbit polyclonal anti-HADHB (Novus Biologicals, NBP1-82609, 1:1000) or goat polyclonal anti-CIRBP (Abcam, ab106230, 1:400) to visualize HADHB and CIRBP respectively, and counterstained using haematoxylin.

Results

In vivo microRNA regulation

MicroRNA microarray was performed in order to determine differential expression of microRNAs after induction of ischemia in vivo. MicroRNAs from the 14q32 cluster showed three different temporal expression patterns after single ligation of the femoral artery. One third of the 14q32 microRNAs were upregulated within 24 hours after ischemia induction (early responders, Figure 1A, average expression Figure 1B). Another third of 14q32 microRNAs were upregulated 72 hours after induction of ischemia (late responders, Figure 1C, average expression Figure 1D), whereas the other 14q32 microRNAs were not differentially expressed after ischemia (non-responders, Figure 1E, average expression Figure 1F). When looking at the distribution of early, late and non-responders on the 14q32 locus, there was no association between the expression profiles of microRNAs and their corresponding gene's chromosomal location (Figure 1G). In addition, baseline expression levels of 14q32 microRNAs were variable and also independent of their corresponding gene's chromosomal location. Because of their proven efficacy in post-ischemic neovascularization[15], we focused on early responder miR-494-3p, late responder miR-329-3p and non-responder miR-495-3p for further experiments.

Pri-miR, pre-miR and mature miR levels of 14q32 microRNAs

Using specific primers for each of the microRNA processing steps of 14q32 microRNAs miR-329-3p, miR-494 and miR-495-3p, we determined the expression levels of the pri-miR, pre-miR and mature microRNA of these microRNAs in the adductor muscle tissue after induction of ischemia in mice. Expression of pri-miR-329 decreased slightly after ischemia induction, but increased in expression by day 7 after induction of ischemia. Pre-miR-329 followed expression of pri-miR-329 at 24 hours after ischemia but continued to decrease until 72 hours after ischemia induction. Expression of mature miR-329-3p mirrored expression of pre-miR-329 (Figure 2A). For early responder miR-494, expression of the pri-miR transcript was also decreased at 24 and 72 hours after ischemia induction. The abundance of pre-miR-494 however was slightly reduced 24 hours after ischemia, whereas mature miR-494 levels were upregulated (Figure 2B). Expression of microRNA-495 pri-miR and pre-miR followed the same pattern after ischemia, showing decreased expression within 24 hours (Figure 2C). However, mature miR-495-3p levels remained unchanged after ischemia induction. Where pre-miR expression levels were exhausted, we observed increased expression of mature miR levels, which was especially true for early responder miR-494 and late responder miR-329-3p. These data suggest that regulation of 14q32 microRNA processing takes places at the conversion from pre-miR to mature microRNA.

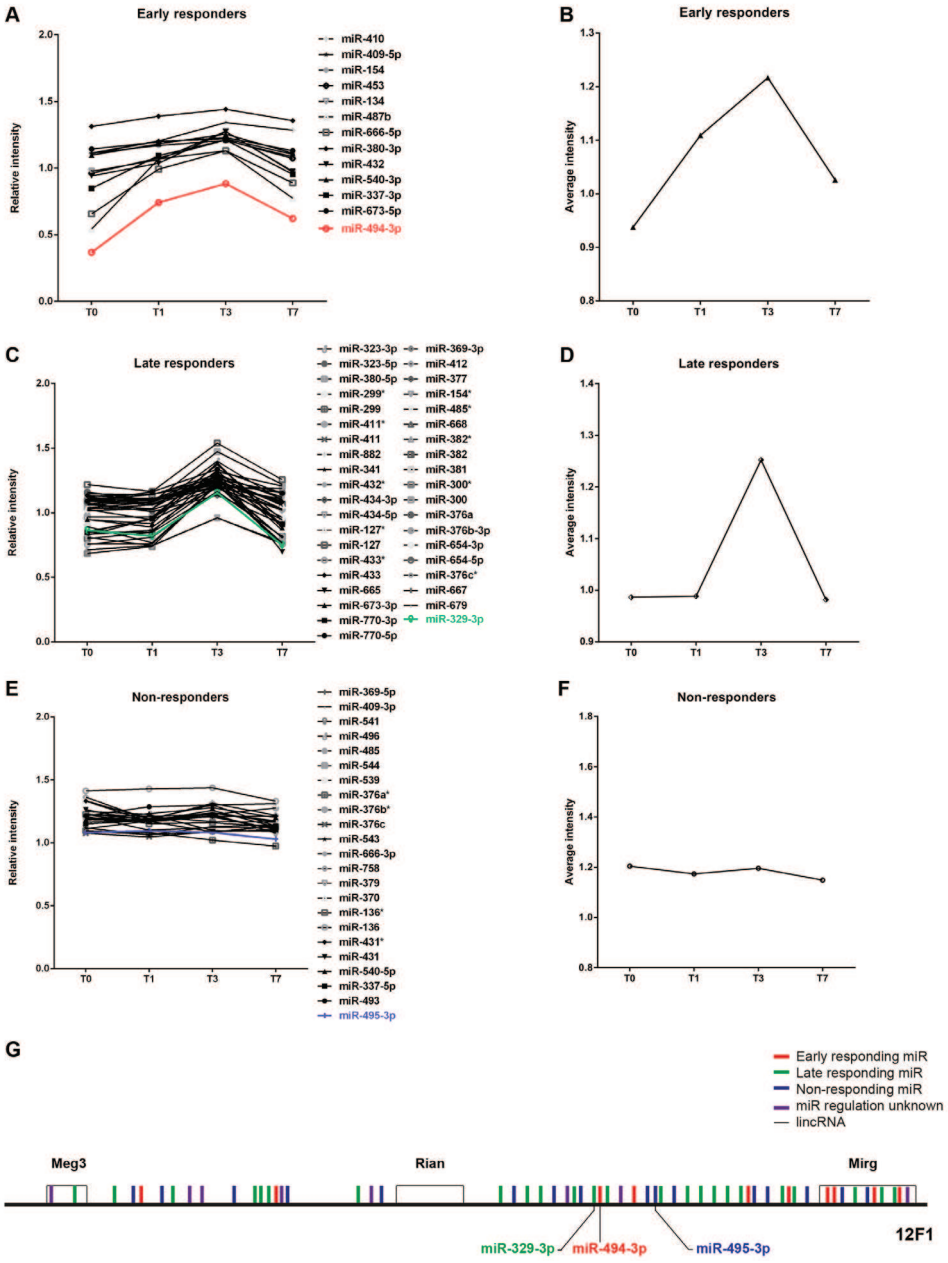


Figure 1. Differential expression patterns of 14q32 microRNAs after induction of ischemia. MicroRNA expression was evaluated before induction of ischemia (T0), day 1 (T1), day 3 (T3) and day 7 (T7) after induction of ischemia. Early responder 14q32 microRNAs were upregulated within 24 hours after ischemia (A). Average intensity of all early responders (B). Late responder 14q32 microRNAs were not upregulated until 72 hours after ischemia induction (C). Average intensity of all late responders (D). Non-responder 14q32 microRNAs were not regulated after ischemia (E). Average intensity of all non-responders (F). Chromosomal location of early (red), late (green) and non-responders (blue) on the murine 12F1 locus (G).

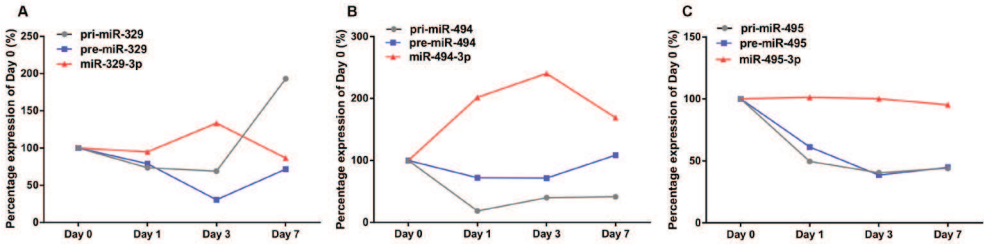


Figure 2. Pri-miR, pre-miR and mature miR expression levels of 14q32 microRNAs. Percentage of expression (relative to day 0) of pri-miR-329, pre-miR-329 and mature miR-329-3p at day 0 (no ischemia), day 1, day 3 and day 7 after ischemia induction (A). Percentage expression of day 0 of pri-, pre- and mature miR-494-3p after ischemia (B). Percentage expression of day 0 of pri-, pre- and mature miR-495 after ischemia (C).

Pre-microRNA pull down followed by SILAC Mass Spectrometry reveals putative 14q32 microRNA biogenesis factors

Using serum-starvation of 3T3 cells, we were able to mimic the *in vivo* regulation of 14q32 microRNAs miR-329-3p and miR-495-3p, but not miR-494-3p *in vitro* (Figure 3). We therefore focussed on miR-329-3p and miR-495-3p. To identify the RBPs that are responsible for differential expression of these two microRNAs via posttranscriptional regulation, we performed Stable Isotope Labelling of Amino Acids, followed by RNA pull down and Mass Spectrometry (Figure 4A-B). We identified several proteins, which were specifically bound to pre-miR-329 and pre-miR-495, namely RBM28 and TBL3 (pre-miR-329 specific), RBM15 (pre-miR-495 specific) as well as proteins CKAP4, P4HB, CIRBP and HADHB, which were bound to both pre-miR-329 and pre-miR-495, showing an increase in binding after serum starvation. Using Western Blot analysis, we validated binding of HADHB and CIRBP to miR-329 and miR-495 precursors (Figure 4C-F). While both proteins seemed to bind pre-miR-495 in both serum starvation and non-starvation conditions, binding to pre-miR-329 was greatly increased following serum starvation.

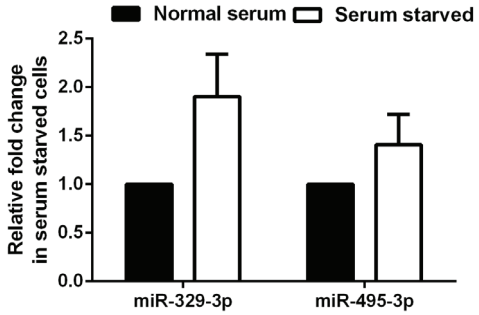


Figure 3. Levels of mature miR-329 and miR-495 in serum starved 3T3 cells. The levels of mature miR-329-3p and miR-495-3p were determined by rt/qPCR. 3T3 cells were serum-starved for 24h and expression levels were compared to non-starved cells.

CIRBP and HADHB expression in vivo

We next determined expression of RNA binding proteins CIRBP and HADHB *in vivo* in the adductor muscle at different timepoints after induction of ischemia. Using immunohistochemistry, we confirmed expression of both CIRBP and HADHB in the adductor muscle of mice after ischemia (Figure 5A and 5B,

respectively). Expression of CIRBP mRNA was increased within 24 hours after induction of ischemia, whereas HADHB mRNA was increased at 3 days after induction of ischemia (Figures 5C and 5D). Finally, using western blot analysis of CIRBP and HADHB in adductor muscle at different time points, we observed an acute but transient increase in CIRBP protein levels at day 1 after ischemia induction. HADHB protein levels were also upregulated within 1 day, but they remained elevated until 10 days after induction of ischemia (Figure 5E and 5F).

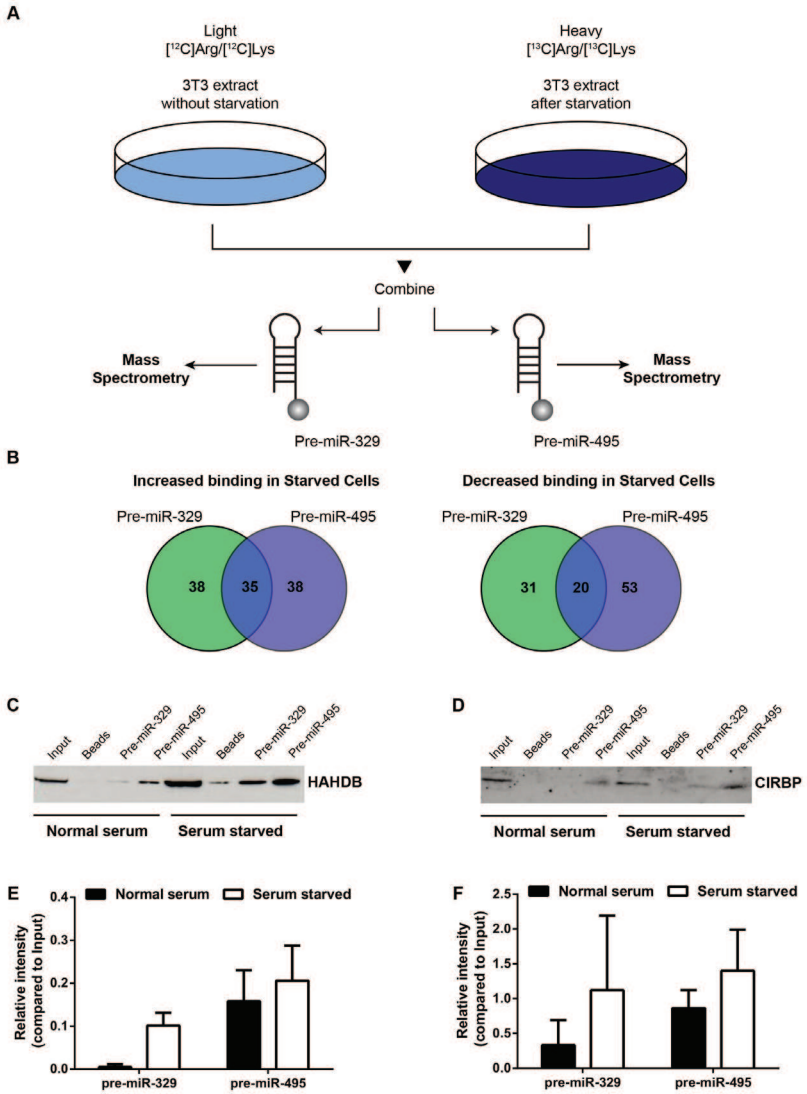


Figure 4. Identification of proteins binding pre-miR-329 and pre-miR-495 (A) Schematic representation of RNA pull down combined with SILAC mass spectrometry (B) Number of proteins showing increased and decreased binding to pre-miR-329, pre-miR-495 or both. Western Blot validation of HADHB (C) and CIRBP (D) binding to pre-miR-329 and pre-miR-495 under conditions of normal serum and serum starvation. (E-F) Western Blot quantification of HADHB (E) and CIRBP (F) binding to pre-miR-329 and pre-miR-495 under conditions of normal serum and serum starve conditions (relative to input (n=3)).

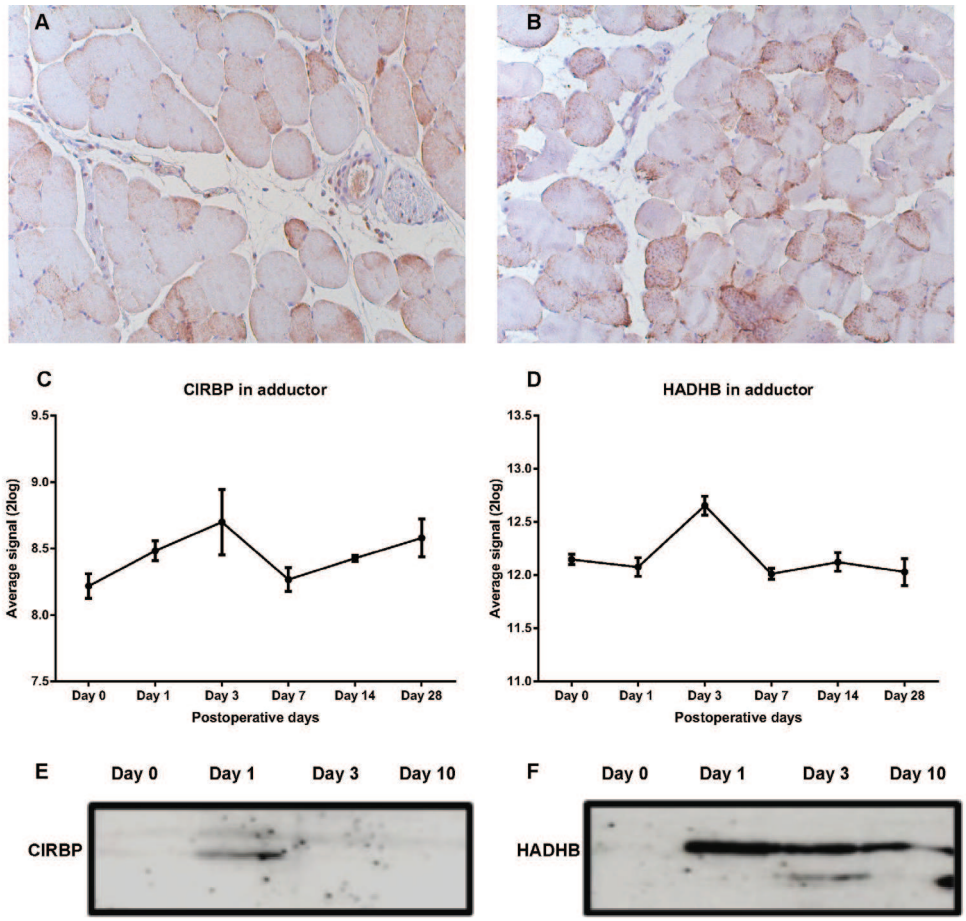


Figure 5. Expression of CIRBP and HADHB *in vivo*. Immunohistochemical staining of the murine adductor muscle after ischemia induction revealed expression of both CIRBP (A) and HADHB (B) in these tissues, predominantly in the cytoplasm of cells. Microarray analysis of mRNA expression of CIRBP (C) and HADHB (D) mRNA in the adductor muscle of mice at several time points after induction of ischemia. (E, F) Western Blot analysis of CIRBP and HADHB protein levels in the adductor muscle at several time points after ischemia.

HADHB and CIRBP binding to miR-329 pri-miR, pre-miR and mature miR transcripts

RIP experiments using antibodies against HADHB and CIRBP or a negative control IgG was performed to determine interaction between RBPs HADHB and CIRBP and 14q32 miR-329 and miR-495 precursors in 3T3 cell cultures. Whereas HADHB binds both pri-miR-329 and pre-miR-329, CIRBP showed specific binding to pre-miR-329, not to pri-miR-329 (Figure 6). CIRBP and HADHB both bind the mature miR-329-3p. Expression of pri-miR-495 and pre-miR-495 was too low to either confirm or exclude binding of CIRBP or HADHB. RIP experiments using an unrelated RBP that has been shown to regulate processing of polycistronic microRNAs, namely SND1[18], showed no specific binding to either pri-miRs or pre-miRs of miR-329 and miR-495 (Supplemental Figure 1).

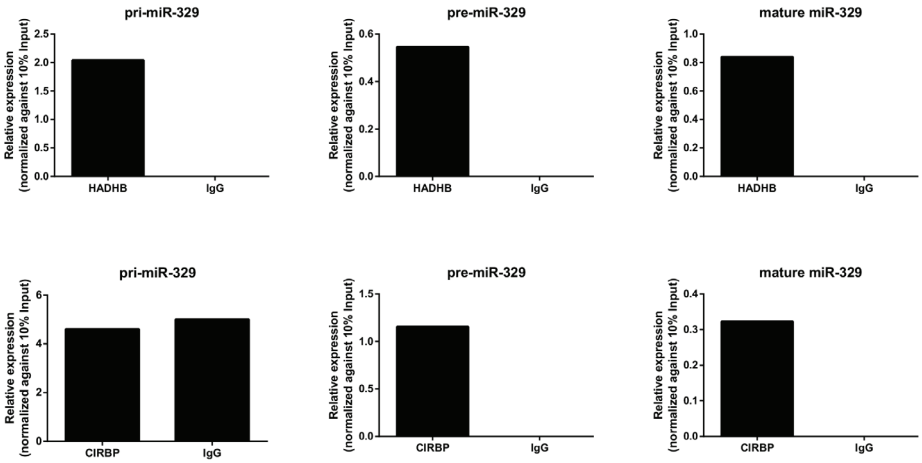
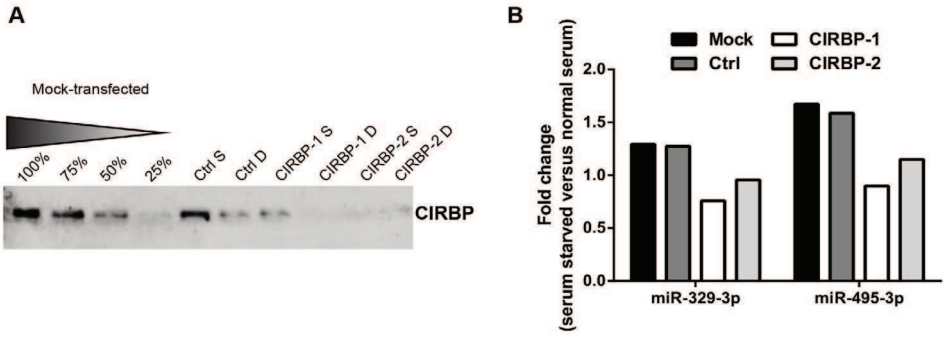


Figure 6. RNA binding protein immunoprecipitation with HADHB and CIRBP antibodies. Pri-miR-329, pre-miR-329 and mature miR-329 expression levels were measured in 3T3 cell lysates after immunoprecipitation with HADHB (top) and CIRBP (bottom) antibodies and non-specific IgG antibody.

Expression of miR-329-3p and miR-495-3p after CIRBP silencing

Next, we used two different siRNAs (CIRBP-1 and CIRBP-2) to knock-down the levels of CIRBP in 3T3 cells. Silencing of CIRBP was performed using either a single or double transfection with CIRBP targeting siRNAs. Both single and double transfections reduced CIRBP protein levels, which was confirmed using Western Blot analysis (Figure 7A). Next, we measured the levels of mature microRNAs miR-329-3p and miR-495-3p in 3T3 cells following 24 hours serum-starvation (Figure 7B). Following serum-starvation, expression of both miR-329-3p and miR-495-3p was downregulated in CIRBP knock-down cells, which was not observed in cells that were either mock transfected or transfected with control siRNA.

Figure 7. siRNA mediated knockdown of CIRBP. Western Blot showing protein levels of CIRBP (A). The first 4 lanes show decreased



concentrations of mock-transfected 3T3 extract, reflecting decreased CIRBP concentrations. The next lanes show CIRBP protein levels in 3T3 cells following either a single (S) or double (D) transfection with siRNAs targeting CIRBP (CIRBP-1 and CIRBP-2) or control siRNA (A). Fold change of mature miR-329-3p or miR-495-3p levels in starved cells after double transfection with siRNAs against CIRBP (B).

Discussion

In this study, we investigated posttranscriptional regulation of 14q32 microRNAs during ischemia. Using specific primers for each of the microRNA processing steps, we found that increased expression of 14q32 microRNAs after hind limb ischemia is not determined by increased transcription, but that it is the result of increased processing of pre-microRNA to mature microRNA. The observed expression pattern of 14q32 microRNAs *in vivo* after ischemia was mimicked *in vitro* using serum starvation of 3T3 cells. This allowed us to investigate which RBPs can bind to pre-miR transcripts of 14q32 microRNAs, using Stable Isotope Labelling of Amino Acids (SILAC), followed by RNA pull-down and Mass Spectrometry. The RNA binding proteins CIRBP and HADHB were shown to specifically bind miR-329 and miR-495 precursors. We confirmed expression and upregulation of these RBPs in murine muscle tissues during ischemia, at mRNA level as well as protein level.

Posttranscriptional regulation of polycistronic microRNAs has been described previously. In fact, differential expression after induction of hindlimb ischemia, similar to that of the 14q32 microRNAs, has also been shown for microRNAs of the polycistronic miR-17-92a cluster. The miR-17-92a cluster encodes for seven mature microRNAs and is transcribed as one single primary transcript[19]. However, individual members of the miR-17-92a cluster were differentially expressed during endothelial differentiation of murine embryonic stem cells[20]. The SND1 protein, which is a component of the RISC, was found to bind to pri-miRs, pre-miRs and mature microRNAs of the miR-17-92a cluster. Silencing of SND1 reduced processing of miR-17-92a cluster members, especially under hypoxic conditions[18]. Here, we could not demonstrate binding of SND1 to either pri-miRs or pre-miRs of 14q32 microRNAs miR-329 and miR-495 using RIP experiments. Our data demonstrate that processing of 14q32 microRNAs miR-329 and miR-495 is independent of SND1 binding, but instead relies on CIRBP and HADHB.

CIRBP is an evolutionary conserved RBP that is transcriptionally upregulated in low temperature conditions or other conditions of stress, including ischemia[21, 22]. CIRBP protein is predominantly expressed in the nucleus, but can also be transported to the cytoplasm under physiological or stressful conditions[23]. MicroRNA processing from pre-miR to mature microRNA also occurs in the cytoplasm. CIRBP is involved in posttranscriptional regulation of mRNAs. Here however, we show for the first time that CIRBP is also involved in posttranscriptional regulation of microRNAs. Future experiments will have to determine whether CIRBP can also regulate other microRNAs during ischemia.

HADHB forms the beta subunit of the mitochondrial trifunctional protein, which catalyzes the last steps of mitochondrial beta-oxidation of long chain fatty acids. In addition, HADHB was found to act as an RBP and bind renin mRNA, leading to destabilization of renin mRNA[24]. Localization of HADHB was found to be predominantly in mitochondria, but also in the cytoplasm and nucleoli of Calu-6 cells[25]. In this study, we observed both cytoplasmic as well as nuclear expression of both HADHB and CIRBP in murine adductor muscle tissue after ischemia. In addition, we have now shown that HADHB can also bind pri-miRs, pre-miRs and mature microRNAs of the 14q32 microRNA cluster, indicating its role in posttranscriptional regulation of microRNA expression under ischemia.

Regulation of microRNA processing under stress conditions such as hypoxia has been previously reported by several studies[26-28]. In endothelial cells, hypoxia was shown to both increase expression of certain microRNAs (such as miR-210)[27] as well as to reduce microRNA processing[28]. Further examination revealed that chronic hypoxia down-regulated expression of Dicer, reducing subsequent microRNA processing[28]. More recently, the involvement of the Epidermal Growth Factor Receptor (EGFR) in microRNA processing under hypoxic conditions was reported. EGFR was shown to increase phosphorylation of AGO2 under hypoxic conditions, which reduced AGO2 binding to Dicer and subsequent microRNA processing from pre-microRNA to mature microRNA by Dicer[29]. Although we did not study regulation of 14q32 microRNA processing under true hypoxic conditions *in vitro*, we were able to demonstrate increased processing under conditions of serum starvation.

In this study, we identified RBPs that bind and regulate specific 14q32 microRNA precursors. These results provide insights into the complex regulation of the 14q32 microRNAs. We showed for the first time that CIRBP and HADHB, which have been shown to control posttranscriptional regulation of mRNAs, are also involved in posttranscriptional processing of microRNAs. Through manipulation of CIRBP and HADHB, that control expression of 14q32 microRNAs, we may be able to influence 14q32 microRNA expression higher up the regulatory cascade, possibly having more profound therapeutic effects on post-ischemic neovascularization.

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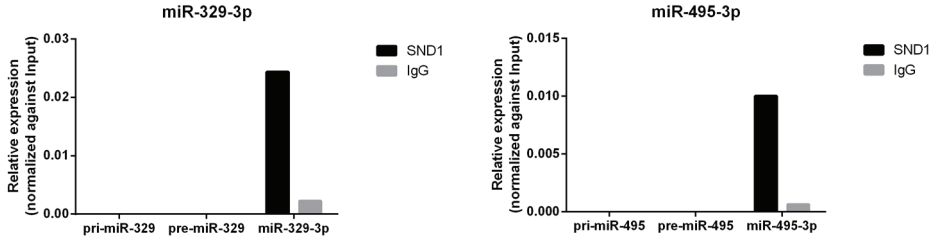
Supplemental Material

Gene	Forward Primer	Reverse Primer
primiR-329	AAGGTCACGTTGGGGAATTA	ACCACGAAGCCTCCAAGAT
premiR-329	TGGTACCGGAAGAGAGGTTTT	AGGTTAGCTGGGTGTGTTTCA
primiR-495	AGCATCCCTTCACACTCAGG	GAGCTCTCCAAGGTGAGATTTG
premiR-495	GTTGCCCATGTTATTTTTCG	AGTGCACCATGTTTGTTTCG
CIRBP	TTTTCGTGGGAGGACTCAGC	CCCTGTCCTTTACCACCACC
HADHB	CAGCGCCTGTCCTTACTCAG	CAGAGTGGCCCATGGTCTC

Supplementary Table 1. List of primers used.



Supplemental Figure 1. RNA binding protein immunoprecipitation with SND1 antibody. Pri-miR, pre-miR and mature microRNA expression levels of miR-329-3p and miR-495-3p were measured in 3T3 cell lysates after immunoprecipitation with SND1 antibody and a non-specific IgG antibody.

Part III

14q32 microRNAs in adipose tissue