Regulation of miR-17-92a cluster processing by the microRNA binding protein SND1

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Article info
Article history:
Received 20 February 2013
Revised 3 June 2013
Accepted 3 June 2013
Available online 13 June 2013

Edited by Tamas Dalmay

Keywords:
MicroRNA
Tudor-SN
Processing
Endothelial

1. Introduction

MicroRNAs (miRs) are small non-coding RNAs that posttranscriptionally control gene expression and play crucial roles in tissue homeostasis and disease [1,2]. The primary transcripts of miRs (pri-miRs) are transcribed by RNA polymerase II and are subsequently processed by the microprocessor complex comprised of the RNase III Drosha, the DiGeorge syndrome critical region gene 8, and the DEAD box RNA helicases p68 and p72 [3]. The cropping of the pri-miR transcript by the Drosha complex results in the formation of miR precursors (pre-miRs), which are exported from the nucleus and are further “diced” into the mature ~22 nucleotide long miRs by the RNase III Dicer together with the double-stranded RNA-binding domain protein TRBP [4].

The expression of miRs is controlled at the transcriptional level, however, increasing evidence supports the critical importance of the posttranscriptional regulation of miR biogenesis. MiR processing can be regulated at the level of cropping by modulating Drosha-dependent processing of pri-miRs to pre-miRs. The concentration of mature miRs is additionally controlled by modulation of Dicer-dependent processing of the pre-miRs to the mature miRs or by mechanisms influencing the stability of the mature miRs [5–7]. Depletion of the RNA helicases p68 and p72 for example has been found to reduce a subset of miRs by blocking the formation of pre-miRs [8]. Upon DNA damage, the transcription factor p53 can bind to the RNA helicase p68 and Drosha and thereby enhances processing of miR-16 and miR-143 [9]. SMAD proteins are another example of proteins that bind to this complex, and it has been demonstrated that stimulation of smooth muscle cells with growth factors promotes SMAD binding to p68 and Drosha thereby controlling pre-miR-21 processing [10]. In addition, miR biogenesis is controlled by editing [11]. For example, editing of pri-miR-142 resulted in degradation by the staphylococcal nuclease domain-containing protein Tudor-SN (SND1), thereby reducing the concentration of the mature miR [11].

The polycistronic miR-17-92a cluster encodes seven mature miRs miR-17-5p (miR-17), miR-17-3p, miR-18a, miR-19a, miR-20a, miR-19b and miR-92a. The miR-17-92a cluster is highly expressed in tumors and was therefore named “OncomiR-1”. Meanwhile various additional functions have been assigned to the cluster including the regulation of hematopoiesis, immune functions and angiogenesis [12–15]. MiR-17-92a deficient mice die during early postnatal development with cardiopulmonary defects [16]. Although the miR-17-92a cluster is transcribed as one
primary transcript, several studies demonstrate that the individual members of the cluster are differentially regulated. For example, during endothelial differentiation of mouse embryonic stem cells the pri-miR-17–92 transcript is increasing while the mature cluster member miR-92a is decreasing [17,18]. Moreover, in medulloblastomas it was observed that miR-19a levels are higher than miR-92a levels [19].

The mechanism underlying the differential posttranscriptional regulation of the individual members of the miR-17-92a cluster is mainly unclear, but it was shown that Drosha dependent processing of pri-miR-18 but not the other members of the family depends on the RNA-binding protein hnRNP A1 [20,21]. Thereby, hnRNP A1 specifically binds to the loop sequence of pri-miR-18 and acts as a chaperone to promote cropping by the microprocessor complex [21].

Here we aimed at the identification of factors binding to pre-miR-92a that potentially act as posttranscriptional regulators. SIRNA mediated silencing of pre-miR-92a binding proteins revealed their impact on the processing of the miR-17-92a cluster.

2. Materials and methods

2.1. Cloning and in vitro transcription of pre-miR-92a

The pre-miR-92a sequence (CUUUCUACACAGGUUGGAUUCG-GUUGCAAGCGUGUUGUAGCUAUUGCUACUCUUGCCGGCCUGU- CUGAGUUG) was cloned with flanking Apc restriction sites into pGEM-T in both, correctly and reversely oriented direction. Pre-miR-92a and the reverse sequence (subsequently named pre-miR-co) were in vitro transcribed by run-off transcription using T7 as described previously [22].

2.2. Cell culture

Human umbilical vein endothelial cells (HUVECs) were purchased from Lonza and cultured in endothelial basal medium (EBM; Lonza, D-Köln) supplemented with hydrocortisone, bovine brain extract, epidermal growth factor, gentamycin sulphate, amphotericin-B, and 10% fetal calf serum (FCS; Life Technologies, D-Darmstadt) until the third passage as previously described [23].

2.3. RNA pulldown

Published protocols for RNA pulldowns [20,24,25] were adapted for the use of endothelial cells. Protein preparation: native proteins from 10⁶ HUVECs were isolated using the AllPrep Kit (Qiagen, D-Hilden) according to the manufacturer’s protocol. Proteins were dialysed in pulldown buffer (20 mM Tris–HCl pH 7.9, 6% (v/v) Glycerin, 0.1 M KCl, 0.2 mM EDTA, 0.5 mM DTT) and concentrated for the pulldown. RNA-bead preparation: 500 pmol in vitro transcribed RNA was resuspended in 100 μl and incubated for 1 h at room temperature. After precipitation, RNA was resuspended in 100 μl 0.1 M NaOAc pH 5.0. In order to immobilize the RNA to beads, 200 μl of 50% slurry adipic acid dihydrazide beads (Sigma, D-Taufkirchen) were washed three times with 1.5 ml 0.1 M NaOAc pH 5.0, resuspended in 300 μl NaOAc pH 5.0 and were incubated with the oxidized RNA over night at 4 °C under rotation. Pulldown: RNA coupled beads were washed three times with 1 ml 2 M KCl and three times with 1 ml pulldown buffer containing 1.5 mM MgCl₂ and twice with 1 ml water. To elute RNA binding proteins, beads were incubated with RNase V1 (5 units), RNase A (2500 units) and RNase T1 (5000 units) in 50 μl elution buffer (20 mM Tris–HCl pH 7.9, 100 mM NaCl, 5 mM MgCl₂) for 30 min at 37 °C under rotation.

2.4. RNA Immunoprecipitation

RIP was performed using the MagnaRIP™ Kit according to the manufacturer’s protocol (Millipore) with antibodies against elf4A2 (ab31218), SND1 (ab65078, Abcam, UK-Cambridge), Ago2 (Anti-Ago2, clone 9E8.2, Millipore, D-Darmstadt) or IgG as negative control. To crosslink RNA and proteins, HUVECs were treated with 0.1% formaldehyde in PBS, for 30 min at 4 °C and subsequently lysed with RIP lysis buffer for 40 min on ice. Antibody coupled beads were incubated with HUVEC lysates (input) over night at 4 °C. After washing and elution, the RNA was purified with phenol–chloroform, ethanol precipitated, and resuspended in water. To ensure efficient binding of the proteins to the antibodies, the presence of the proteins was monitored in the input, as well as in the supernatant after incubation with the antibody coupled beads. The input fraction was kept under the same conditions as the beads to enable comparison with the proteins used for the RIP.

2.5. Mass spectrometry

Eluted proteins from the RNA pulldown were separated via SDS–PAGE (4–12% Novex-gels, Invitrogen, D-Darmstadt) and stained with colloidal Coomassie. The whole lane of the control and pre-miR-92a pulldown were excised and subjected to in gel-digestion with trypsin. Proteins were measured by mass spectrometry with an LTQ-Orbitrap XL as previously described [26] and label free protein quantification was performed with the MaxQuant Software tool [27].

2.6. siRNA transfection and deferoxamin (DFO) treatment

HUVECs were transfected with 40 nM siRNA using Lipofectamine RNAiMax according to the manufacturer’s protocol (Life Technologies, D-Darmstadt). Predesigned siRNA targeted the following sequences (Qiagen, D-Hilden): sielf4A2 5’-TTGCTCAAGCTCAGTCAAGTA-3’, sisiND1 5’-ATCCACCAGTGTTGAGATATA-3’, siScr (targeting firefly luciferase as control) 5’-CGTACCGGAATACCTTCGA-3’. Twenty … hour 5’-CGTACCGGAATACCTTCGA-3’. Twenty four hours after transfection the cells were treated with 100 μM DFO (Sigma, D-Taufkirchen). 96 h after transfection RNA was isolated from the cells using Trizol (Sigma, D-Taufkirchen) and Chloroform, precipitated with isopropanol and resuspended in water.

2.7. qRT-PCR

For analysis of miR expression by quantitative real time PCR (qRT-PCR) 10 ng RNA isolated from HUVECs were reverse transcribed in a total volume of 15 μl using Taqman miRNA assays (Life Technologies, D-Darmstadt). SnoRD48 (U48) was used as a control. RNA eluted from RIP had a concentration below the measurable values, so that 5 μl were reverse transcribed. 1.4 μl of the product was used as template for the qRT-PCR in a StepOne Plus real-time PCR thermocycler. For quantification of miRNA, pri-miR and pre-miR expression, cDNA synthesis was performed with 500 ng RNA using MuLV reverse transcriptase (Applied Biosystems, US-Carlsbad) in a 20 μl reaction. After diluting to a final volume of 200 μl, SYBR green qRT-PCR was performed with 8 μl cDNA. RPLP0 served as a house keeping control. Primer sequences are listed in Supplementary Table 1.
2.8. Statistical analysis

The relative expression levels of two treatment groups were compared using unpaired student’s t-test and GraphPad prism software. A significance level of $P < 0.05$ was considered significant. Data are presented as mean with error bars depicting the standard error of the mean (SEM).

3. Results

3.1. Identification of pre-miR-92a binding proteins

To identify pre-miR-92a binding proteins, pre-miR-92a was in vitro transcribed and immobilized to agarose beads. Pre-miR-92a conjugated beads were then incubated with protein extracts that were generated of HUVECs (Fig. 1a). After extensive washing, bound proteins were eluted by RNase digestion. To determine the specificity of the binding, the pre-miR-92a sequence was used in reversed orientation (pre-miR-co). Eluted proteins were subjected to mass spectrometry (Fig. 1b). Overall, 192 proteins were identified and 59 proteins were found to be enriched in the pre-miR-92a eluate compared to the control experiment. Several proteins were eluted from both the pre-miR-92a and pre-miR-co conjugated beads including potential contaminating proteins such as keratins (data not shown). Among the proteins that were enriched in the pre-miR-92a eluate, 29 are known to bind nucleotides and 12 proteins have a described RNA binding activity (Table 1, and Fig. 1c). Among the highly enriched RNA binding proteins in the pre-miR-92a eluate are the eukaryotic initiation factor 4A-II (eIF4A2) and SND1 (Fig. 1d).

In order to confirm the interaction between pre-miR-92a and eIF4A2 and SND1, we performed RIP experiments to precipitate eIF4A2- and SND1-bound RNAs. Ago2 RIPs were used as controls (Supplementary Fig. 1). EIF4A2- and SND1-RIPs were subjected to Western blotting confirming that both proteins are specifically precipitated (Fig. 2a). SND1 was confirmed as a protein interacting with pre-miR-92a, whereas we did not detect an association with eIF4A2 (Fig. 2b). SND1 but not eIF4A2 additionally interacted with the pri-miR-17-92 transcript (Fig. 2c). Both proteins interact with the mature members of the miR-17-92a cluster miR-17, miR-18a, miR-19a, miR-20a, miR-92a (Fig. 2d).

3.2. eIF4A2 does not regulate the biogenesis of the miR-17-92a cluster members

Since eIF4A2 was shown to bind to the mature members of the cluster, we determined whether eIF4A2 might control the expression of the mature miRs of the cluster (Fig. 2). However, silencing of eIF4A2 (Fig. 3a) did not affect the expression of pri-miR-17-92, pre-miRs, or miR-17, miR-18, miR-19, miR-20, and miR-92a (Fig. 3b–d). In addition, inhibition of eIF4A2 expression under conditions that mimic hypoxia (DFO treatment) did not affect the expression of pri-, pre-, or mature miR-17-92a cluster members and only slightly reduced all mature miR-17-92a family members (data not shown).

Fig. 1. Identification of miR-binding proteins. (a) Pre-miR-92a was in vitro transcribed, oxidized and bound to adipic acid dihydrazide beads and incubated with 1 mg of HUVEC proteins. To monitor the efficacy of the RNA binding to the beads, beads were washed four times and RNA was eluted by heat (5 min, 95 °C). 20 μl was loaded on a 10% TBE gel (Invitrogen, D-Taufkirchen). w = washing fraction, M = low range ssRNA marker (NEB, D-Frankfurt). (b) Twelve percentage SDS–PAGE with proteins bound to pre-miR-92a or a pre-miR-co after elution with RNases. M = page ruler prestained protein ladder (Thermo Scientific). (c) In total, 192 proteins were identified by mass spectrometry. 31% are nucleic acid binding, but not explicitly RNA binding. 11% are known RNA binding proteins. (d) Highly enriched pre-miR-92a bound proteins known to interact with nucleic acids.
3.3. **SND1 modulates the expression of the miR-17-92a cluster members**

Since SND1 was shown to bind to the pre-miR-92a as well as to the mature members of the miR-17-92a cluster (Fig. 2), we explored whether silencing of SND1 may affect the processing of the cluster. SND1 was efficiently silenced by siRNA (Fig. 4a). Under basal conditions, the expression of the pri-, pre-miRs and mature miRs was not affected (Fig. 4b–d). However, under stress conditions imposed by the hypoxia mimicking iron chelator DFO, silencing of SND1 modulated miR-17-92a expression. DFO enhanced the expression of pri-miR-17-92 as well as pre-miR-17, pre-miR-20 and pre-miR-92a. Despite an increase in the pre-miRs, DFO did not increase the expression of the mature miR-17-92a cluster members (Fig. 4d) suggesting that mimicking hypoxia interferes with processing of the precursor miRs. Silencing of SND1 reduced

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#### Table 1

<table>
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<th>Protein specifically bonding to pre-miR-92a</th>
<th>Gene</th>
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<th>pre-miR-92a</th>
<th>Unique peptides ctrl</th>
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<td>9 10</td>
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<td>n.d.</td>
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<td>n.d.</td>
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<td>1.3E+05</td>
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Grey: RNA binding proteins, white: nucleic acid binding but not RNA binding. Cut off for valid proteins was set to normalized intensity >5 x 10^4. Ctrl = pre-miR-co.
the DFO-mediated increased expression of the pri-miR-17-92 and the precursors (Fig. 4b and c). In addition, SND1 silencing reversed the DFO-mediated inhibition of mature miR formation leading to an increased expression of the mature cluster members miR-17, miR-18, miR-19, miR-20 and miR-92a at 96 h after DFO treatment (Fig. 4d). Thus silencing of SND1 resulted in a significantly reduced ratio of pre-miRs to mature miRs (Fig. 4e).

3.4. The effects of SND on the processing of the miR-17-92a cluster are independent on A to I editing

Since SND1 is known to degrade edited primary miR transcripts and precursors [28], we further explored whether the miR-17-92a cluster might be edited under hypoxia mimicking conditions. However, only very few editing sites were identified even if SND1 was silenced to prevent the potential degradation (Supplementary Fig. 2). Another possible mechanism is based on the observation that SND1, is localized to stress granules after a stress induction did not reveal differences in editing of the pri-miR-17-92a cluster under hypoxia mimicking conditions in the absence of SND1 (Supplementary Fig. 2). Another possible mechanism is based on the observation that SND1, is localized to stress granules after a stress
stimulus [35]. It is conceivable that under DFO treatment, SND1 is localized to stress granules and thereby carries its bound pre-miRs into these compartments, where they cannot be processed. When SND1 is silenced, the pre-miRs have access to the processing machinery in the cytoplasm. This is one speculative mechanism which needs confirmation by further experiments.

The effects of SND1 on the processing of the miR-17-92a cluster may contribute to the regulation of angiogenesis. Silencing of SND1 was shown to inhibit angiogenesis in a chicken chorioallantoic membrane assays and in endothelial cells [36]. Here we describe that silencing of SND1 particularly under hypoxia augments the expression of the mature miR-17-92a cluster members. All mature members of the miR-17-92a cluster were shown to block sprouting and tube formation of endothelial cells in vitro [37]. Moreover, several of the mature members – particularly miR-17/20 and miR-92a – inhibit the vessel growth in vivo [37]. Thus, the increased levels of the anti-angiogenic mature members of the miR-17-92a cluster observed after SND1 silencing in the present study may well fit with the described inhibition of angiogenesis by siRNA mediated SND1 depletion [36].

Fig. 4. Silencing of SND1 under basal and hypoxia mimicking conditions. (a) Knock down efficiency of siRNA against SND1 after 96 h in HUVECs under normoxic (−DFO) and hypoxia mimicking (+DFO) conditions. (b–d) Effect of SND1 silencing after 96 h on pri-miR-17-92 (b), pre-miR (c) and mature miR levels (d). (e) Effect of SND1 silencing on the ratio of pre-miRs to mature miRs under normoxic and hypoxia mimicking conditions. Data are depicted as mean ± S.E.M. n = 3–7, * = P < 0.05, ** = P < 0.01. DFO = Deferoxamin, 100 μM.

Acknowledgements
E.-M. H. is supported by a fellowship of the GRK1172 (DFG). This study was supported by DFG (SFB902 www.SFB902.de Project B2 Dimmeler).

Appendix A. Supplementary data
Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.febslet.2013.06.008.

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