ACTA ABP BIOCHIMICA POLONICA

Vol. 63, No 4/2016
773–783
https://doi.org/10.18388/abp.2016_1339

Regular paper

How short RNAs impact the human ribonuclease Dicer activity: putative regulatory feedback-loops and other RNA-mediated mechanisms controlling microRNA processing

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Ribonuclease Dicer plays a pivotal role in RNA interference pathways by processing long double-stranded RNAs and single-stranded hairpin RNA precursors into small interfering RNAs (siRNAs) and microRNAs (miRNAs), respectively. While details of Dicer regulation by a variety of proteins are being elucidated, less is known about non-protein factors, e.g. RNA molecules, that may influence this enzyme's activity. Therefore, we decided to investigate the question of whether the RNA molecules can function not only as Dicer substrates but also as its regulators. Our previous *in vitro* studies indicated that the activity of human Dicer can be influenced by short RNA molecules that either bind to Dicer or interact with its substrates, or both. Those studies were carried out with commercial Dicer preparations. Nevertheless, such preparations are usually not homogeneous enough to carry out more detailed RNA-binding studies. Therefore, we have established our own system for the production of human Dicer in insect cells. In this manuscript, we characterize the RNA-binding and RNA-cleavage properties of the obtained preparation. We demonstrate that Dicer can efficiently bind single-stranded RNAs that are longer than ~20-nucleotides. Consequently, we revisit possible scenarios of Dicer regulation by single-stranded RNA species ranging from ~10- to ~60-nucleotides, in the context of their binding to this enzyme. Finally, we show that siRNA/miRNA-sized RNAs may affect miRNA production either by binding to Dicer or by participating in regulatory feedback-loops. Altogether, our studies suggest a broad regulatory role of short RNAs in Dicer functioning.

Key words: ribonuclease Dicer; miRNA processing; regulatory RNAs; regulation of Dicer activity; regulatory feedback-loops

Received: 01 June, 2016; revised: 27 June, 2016; accepted: 30 June, 2016; available on-line: 13 October, 2016

INTRODUCTION

One of the key components of RNA interference (RNAi) pathways is a multi-domain ribonuclease (RNase), called Dicer. Dicer is responsible for processing of double-stranded RNAs (dsRNAs) into small interfering RNAs (siRNAs) and excision of miRNAs from their hairpin precursors (pre-miRNAs) (Bernstein *et al.*, 2001). In humans, miRNAs are the most abundant and as yet the best characterized group of small regulatory RNAs. They have been found to regulate the expression of the majority of protein-coding genes through the miRNA pathway (Friedman *et al.*, 2009). It has been also demonstrated that miRNAs play a very important role in the interplay between the host and the virus (Berkhout & Haasnoot, 2006; Haasnoot & Berkhout, 2006; Kurzynska-Kokorniak *et al.*, 2009; Jackowiak *et al.*, 2011a). Consequently, the cellular levels of miRNAs and other components of miRNA pathways must be tightly controlled. Deregulation of miRNA levels can initiate pathological processes, including carcinogenesis, neurodegenerative, immune system and rheumatic disorders (Calin & Croce, 2006; Esquela-Kerscher & Slack, 2006; Tili *et al.*, 2008; Hebert & De Strooper, 2009).

Human Dicer is a ~220-kDa protein consisting of an N-terminal domain homologous to the DExD/H helicase, a domain of unknown function (DUF 283), the Piwi-Argonaute-Zwille (PAZ) domain, two RNase III domains (RNase IIIa and IIIb) and a C-terminal dsRNA-binding domain (dsRBD) (Bernstein *et al.*, 2001; Zhang *et al.*, 2002; Zhang *et al.*, 2004; Macrae *et al.*, 2006a; Macrae *et al.*, 2006b). Comprehensive structural and biochemical analyses of Dicer have revealed the functions of its individual domains and allowed to propose mechanisms of Dicer functioning. According to the current model, miRNA and siRNA precursors are predominantly recognized by the PAZ domain (Yan *et al.*, 2003; Ma *et al.*, 2004; Zhang *et al.*, 2004; Tian *et al.*, 2014). In addition, RNA binding is supported by the dsRBD (Zhang *et al.*, 2004; Ma *et al.*, 2012; Wostenberg *et al.*, 2012). The helicase domain interacts with the terminal loop of pre-miRNAs to align the substrate to the catalytic center of the enzyme for precise cleavage, and to discriminate between miRNA and siRNA precursors (Tsutsumi *et al.*, 2011; Gu *et al.*, 2012; Ma *et al.*, 2012; Taylor *et al.*, 2013). The helicase domain also plays an important regulatory role; it has been shown to be responsible for autoinhibition of Dicer (Ma *et al.*, 2008) and to serve as a binding platform for auxiliary proteins (Bennasser & Jeang, 2006; Daniels *et al.*, 2009), as reviewed in Kurzynska-Kokorniak *et al.* (Kurzynska-Kokorniak *et al.*, 2015). Two RNase III domains of Dicer form an intramolecular

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Abbreviations: dsRNA, double-stranded RNA; hDicer, recombinant human Dicer protein; miRNA, microRNA; miR-Reg, an oligonucleotide with a sequence identical to a specific miRNA; pre-miRNA, pre-microRNA (microRNA precursor); RNAi, RNA interference; siRNA, small interfering RNA; ssRNA, single-stranded RNA

dimer; each domain cleaves the opposing strand of the substrate. This way, ~20-base pair (bp) dsRNA products with characteristic 2-nucleotide (nt) 3' overhangs are generated. Finally, DUF283 has been found to be responsible for interactions with Dicer protein partners (Ota *et al.*, 2013). Moreover, it has been demonstrated that the separate DUF283 domain of Dicer selectively binds single-stranded RNAs (ssRNAs) *in vitro,* and accelerates annealing of complementary nucleic acids (Kurzynska-Kokorniak *et al.*, 2016).

Since the discovery of Dicer in 2000 (Bernstein *et al.*, 2001), much attention has been placed on its role in RNAi/miRNA biogenesis pathways. Dicer is believed to participate in both, the cleavage of siRNA/miRNA precursors and the subsequent translocation of the generated duplexes to the RNA-induced silencing complex (RISC) (Gregory *et al.*, 2005; Maniataki & Mourelatos, 2005; Lee *et al.*, 2006; MacRae *et al.*, 2008; Noland *et al.*, 2011). The substrate specificity of Dicer has been well characterized (Provost *et al.*, 2002; Vermeulen *et al.*, 2005; Lima *et al.*, 2009; Chakravarthy *et al.*, 2010; Tsutsumi *et al.*, 2011; Feng *et al.*, 2012). It has been demonstrated that Dicer binds ss- and dsRNAs with different affinities, depending on their sequence and length (Lima *et al.*, 2009). In addition, RNA-binding and cleavage by Dicer have been shown to depend on the structure of the RNA substrates and the accessibility of their 3'- and 5'-ends (Vermeulen *et al.*, 2005; Chakravarthy *et al.*, 2010; Feng *et al.*, 2012). Dicer activity is subjected to regulation by various protein and non-protein factors, as reviewed in Kurzynska-Kokorniak *et al.* (Kurzynska-Kokorniak *et al.*, 2015). While the first group is relatively well characterized, still little is known about the non-protein factors that may regulate Dicer functioning. The latter group includes RNA molecules that bind to Dicer or to its substrates. For example, Dicer is supposed to interact with messenger RNAs (mRNAs) within so-called 'passive sites' (Rybak-Wolf *et al.*, 2014). These sites are usually located in the coding sequences and the 3' untranslated regions of mRNAs that adopt stem-loop structures. Dicer has been shown to bind but not to cut passive sites. Therefore, passive binding has been proposed to serve either as an anchoring mechanism for the efficient assembly of Dicer-associated protein complexes or as a buffering system that controls the catalytic activity of Dicer by sequestering it from other targets. Interestingly, it has been demonstrated that viruses employ a similar strategy to mislead the host defense mechanisms (Lu & Cullen, 2004; Andersson *et al.*, 2005). For example, adenoviruses produce high amounts of long self-complementary transcripts that compete with endogenous Dicer substrates for binding to the enzyme. Thereby, other viral transcripts are protected from cleavage. *In vitro* studies conducted by our group have also indicated that the activity of human Dicer can be influenced by short RNA molecules that either bind to Dicer or interact with its substrates, or both (Tyczewska *et al.*, 2011; Kurzynska-Kokorniak *et al.*, 2013). Those studies were carried out with commercially available Dicers. However, we have found that commercial Dicer preparations are not homogeneous enough to carry out extended RNA-binding studies; therefore we have established our own system for the production of Dicer in insect cells.

In this manuscript, we report on the production of a new construct expressing a recombinant human Dicer protein (hDicer) in insect cells. To provide an increased efficiency of expression in the baculovirus system, the distance between the promoter and the translation start codon was shortened. The resulting construct lacked an extensive fragment of the 5' untranslated region of the hDicer transcript, which comprised additional AUG start codons. We further characterized the RNase and RNA-binding properties of the obtained preparations. We showed that our hDicer preparations process human pre-miRNAs with efficiency comparable to the commercial enzymes. We also demonstrated that the produced hDicer efficiently binds ssRNAs longer than ~20-nt. Then, we revisited the possible scenarios of regulation of hDicer activity by ssRNAs, ranging from ~10- to ~60-nt, in the context of their binding to the enzyme. Finally, we demonstrated that siRNA/miRNA-sized ssRNAs may affect miRNA production either by binding to hDicer or by regulatory feedback-loops. Our findings strongly support observations that Dicer might be subjected to an RNA-dependent regulation *in vivo*.

MATERIALS AND METHODS

Oligonucleotides. Primers for hDicer cDNA cloning and RNA oligonucleotides used for cleavage assays were purchased from FutureSynthesis. Sequences of all oligonucleotides are listed in Table 1.

The 5'-end labeling of oligonucleotides. The 5'-^{32}P oligonucleotide labeling by T4 Polynucleotide Kinase (Promega) was performed as described previously (Tyczewska *et al.*, 2011; Kurzynska-Kokorniak *et al.*, 2013). The ^{32}P-labeled oligonucleotides were purified by polyacrylamide gel electrophoresis (PAGE), in 8% denaturing gels, and resuspended in water to final concentration of approximately 10 000 cpm/μl.

Preparation of the recombinant human Dicer protein. To prepare hDicer, full-length cDNA encoding transcript variant 2 of *DICER1* (NM_030621), cloned into *MluI* and *NotI* restriction sites of pBlueScript vector, was purchased from GeneCopoeia. To obtain pBS-Dicer-Δ5'UTR, a 238 bp region corresponding to the *DICER1* 5'UTR, located upstream from the ATG selected as an initiation codon, was removed by PCR with the primer set: Δ5'UTR-F/Δ5'UTR-R. The PCR product was subsequently cloned into *Acc65I*/*NsiI* restriction sites. The start codon was disrupted with ORF maintenance (using ΔATG-F/Δ5'UTR-R primer set), and the subsequent substitution in pBS-Dicer-Δ5'UTR was performed using *SalI* and *NsiI* restriction sites.

The recombinant baculovirus expressing N-terminally His6-tagged hDicer was generated using the Bac-to-Bac Baculovirus Expression System (Invitrogen) according to the manufacturer's instructions. The target pFastBac HtB vector was prepared by cloning of the entire sequence of the ATG-less *DICER1* gene into *SalI* and *NotI* restriction sites. The cDNA sequence of the modified Dicer was confirmed by sequencing. Next, the prepared plasmid was transformed into DH10Bac™ *E. coli* competent cell, which yielded the recombinant bacmid DNA that was subsequently used for transfection of Sf9 insect cells using Cellfectin® (Invitrogen). The obtained baculovirus was used to produce hDicer in the Sf9 cells. For expression of hDicer, the Sf9 cells (2×10^6/ml) were infected with the recombinant baculovirus at multiplicity of infection 1.0 (virus:cell ratio 1:1). Cells were collected after 2 days, precipitated and resuspended in ice-cold Ni100 lysis buffer (50 mM Tris-HCl pH 7.5, 100 mM NaCl, 10% glycerol and 0.5% Triton X-100) containing 1x protease inhibitor mix without EDTA (Roche). After 15 min incubation on ice, lysates were centrifuged at $17\,000\times g$ for 10 min at 4°C. The supernatant was incubated on a rotator with nickel-nitrilo triacetic agarose beads

Table 1. The list of oligonucleotides used in this study

Name	sequence (5′→3′)
Δ5′UTR-F	GGGGTACCGTCGACATGAAAAGCCCTGCTTTG
Δ5′UTR-R	GGCGACATAGCAAGTCATAATGAGAACCTGGTG
ΔATG-F	ACGCGTCGACGAAAAGCCCTGCTTTG
RNA12	AGCUUAUCAGAC
RNA14	GGGUACCACCAGAA
RNA22 (miR-33a-3p)	CAAUGUUUCCACAGUGCAUCAC
RNA32	GUGCAUUGUAGUUGCAUUGCAUGUUCUGGUCA
RNA42	GGGAGAAUCAUAAGUAGCCUCCCCCCAUGUUAACAGUUAGCC
RNA52	GGGAGAAUCAUAAGUAGCCCCUCGUUCACUCCCCCCAUGUUAACAGUUAGCC
RNA62	UAGCAGCACGUAAAUAUUGGCGUUAAGAUUCUAAAAUUAUCUCCAGUAUUAACUGUGCUGCU
pre-mir-16-1	UAGCAGCACGUAAAUAUUGGCGUUAAGAUUCUAAAAUUAUCUCCAGUAUUAACUGUGCUGCUGAA
pre-mir-21	AGCUUAUCAGACUGAUGUUGACUGUUGAAUCUCAUGGCAACACCAGUCGAUGGGCUGU
pre-mir-33a	GUGCAUUGUAGUUGCAUUGCAUGUUCUGGUGGUACCCAUGCAAUGUUUCCACAGUGCAUC
pre-mir-210	GCCCCUGCCCACCGCACACUGCGCUGCCCCAGACCCACUGUGCGUGUGACAGCGGCUG
Reg-21-5p (miR-21-5p)	UAGCUUAUCAGACUGAUGUUGA
Reg-21-3p (miR-21-3p)	CAACACCAGUCGAUGGGCUGU
Reg-33a-5p (miR-33a-5p)	UGUCAUUGUAGUUGCAUUGCA
Reg-33a-3p (miR-33a-3p)	CAAUGUUUCCACAGUGCAUCAC

(Ni-NTA, Qiagen) that were pre-washed with Ni100 buffer (resin:batch ratio 1:5). After 14h incubation at 4°C, the beads were packed into a column and successively washed with 10 resin volumes of Ni100 buffer and 10 resin volumes of Ni100 buffer supplemented with 10 mM of imidazole. The bound hDicer was eluted with 3.33 resin volumes of Ni100 buffer containing 250 mM imidazole. The fraction collected was loaded onto Amicon filters (Milipore) and the buffer was exchanged for Q100 (20 mM Tris-HCl pH 7.5, 100 mM NaCl, 10% glycerol). Next, the sample was applied onto HiTrap Q HP column (GE Healthcare) equilibrated with Q100 buffer. The column was consecutively washed with 5 volumes of Q100 buffer and 5 volumes of Q200 buffer (buffer Q100 containing 200 mN NaCl). The protein was eluted with 5 column volumes of buffer Q450 (buffer Q100 containing 450 mN NaCl), and collected in 0.5 ml fractions. Based on the results from SDS-polyacrylamide gel electrophoresis (PAGE) analysis followed by Coomassie Blue staining, the fractions of the highest purity were identified and pooled. Amicon filters (Milipore) were used for buffer exchange and for protein concentration. hDicer preparations were stored in 20 mM Tris-HCl pH 7.5, 50 mM NaCl, 30% glycerol, at –80°C.

hDicer immunoblot analysis. The protein suspensions were analyzed by SDS-PAGE followed by immunoblotting. For immunoblotting, two types of antibodies were used: mouse monoclonal (13D6) against human Dicer (Abcam) or rabbit polyclonal against the His6 tag (Abcam). Immunoreactive proteins were visualized using horseradish peroxidase (HRP) conjugates and enhanced chemiluminescence (ECL) reagent.

hDicer binding assay. The ability of hDicer to bind ssRNA was tested by an electrophoretic mobility shift assay (EMSA). All reactions were carried out in a final volume of 10 μl. hDicer (500 nM, unless stated otherwise) or 1U of commercially available enzyme (Ambion/GenLantis/Invitrogen) was added to 10 000 cpm of ^{32}P-labeled RNA and incubated in a binding buffer (250 mM NaCl, 20 mM Tris-HCl, pH 7.5) for 30 min on ice. After incubation, 2 μl of 60% glycerol with 0.04% bromophenol and 0.04% xylenecyanol were added and the samples were separated on 5% native polyacrylamide gel at 4°C in 1×TBE running buffer. The data were collected using a Fujifilm FLA-5100 Fluorescent Image Analyzer and quantified using MultiGauge 3.0 software (Fujifilm).

hDicer cleavage assay. To investigate hDicer RNase activity, 10 000 cpm of ^{32}P-labeled pre-miRNA substrate was incubated with 200 nM hDicer preparation for 2 h at 37°C, unless stated otherwise. The assay was performed in a final volume of 10 μl, in the standard reaction buffer (250 mM NaCl, 2.5 mM $MgCl_2$ and 20 mM Tris-HCl, pH 7.5). In addition, a reaction mixture without hDicer added was prepared as a control. Furthermore, in controls

including EDTA, the reaction buffer was supplemented with the chelating agent to the final concentration of 50 mM. All reactions involving commercially available hDicers were carried out in the abovementioned buffer using 1U of the enzyme. The applied incubation conditions were the same as for the tested preparations of hDicer. The reactions were stopped by the addition of 1 volume of 8 M urea loading buffer and heating for 5 min at 95°C; the samples were separated on a 15% polyacrylamide/8 M urea gel. The data were collected using a Fujifilm FLA-5100 Fluorescent Image Analyzer and quantified using MultiGauge 3.0 software (Fujifilm).

hDicer cleavage inhibition assay. To test the influence of the selected oligonucleotides on hDicer RNase activity, standard reactions of pre-miRNA cleavage were performed (in a final volume of 10 µl). For each oligonucleotide, three reactions were prepared with the same amount of hDicer (200 nM) and pre-miRNA (10 000 cpm, ~1 pmol) and various amounts of the tested oligonucleotides. The molar ratio of pre-miRNA to oligonucleotide was as follows: 1:1, 1:10, and 1:100. Moreover, two reaction mixtures were prepared as controls: the first one without oligonucleotide added, and the second without hDicer. All reactions were carried out in a standard reaction buffer and were incubated for 2 h at 37°C. In addition, in experiments involving miR-regulators, reaction mixtures were pre-incubated for 15 min in the buffer lacking Mg^{2+} ions. After that time, Mg^{2+} ions were added to induce hDicer cleavage and all samples were further incubated for 2 h at 37°C. The reactions were stopped by addition of 1 volume of 8 M urea loading buffer and heating for 5 min at 95°C; the samples were separated on a 15% polyacrylamide/8 M urea gel. The data were collected using a Fujifilm FLA-5100 Fluorescent Image Analyzer and quantified using MultiGauge 3.0 software (Fujifilm). The efficiency of miRNA production upon addition of the tested oligonucleotide was calculated with respect to the level of miRNA generated in the control reaction, without the oligonucleotide.

Annealing assay assisted by hDicer. The pre-miRNA and oligonucleotide complex formation was analyzed using an electrophoretic mobility shift assay (EMSA). The specific pair of RNAs (pre-miRNA and miR-regulator) was incubated with or without hDicer (200 nM) in a binding buffer (250 mM NaCl, 20 mM Tris-HCl, pH 7.5) for 15 min at 37°C. In the control reactions, no oligonucleotide was added. The reactions were stopped by the addition of SDS to a final concentration of 0.2% and separated by native gel electrophoresis on a 12% polyacrylamide gel. As a molecular mass ladder, the ^{32}P-labeled pre-miRNA (10 000 cpm, 1 pmol,) and the indicated oligonucleotide (100 pmol) were mixed in a binding buffer, heated for 3 min at 95°C, and then slowly cooled to 37°C, to facilitate annealing. The data were collected using a Fujifilm FLA-5100 Fluorescent Image Analyzer and quantified using MultiGauge 3.0 software (Fujifilm).

Bioinformatic analysis. The predictions of RNA structure were performed using servers from the RNAstructure Web Servers family (Bellaousov *et al.* 2013): the bifold server (Mathews *et al.* 1999a; Reuter and Mathews 2010) and the Fold server (Mathews *et al.* 1999b; Reuter & Mathews 2010) with default set of input parameters for each structure prediction.

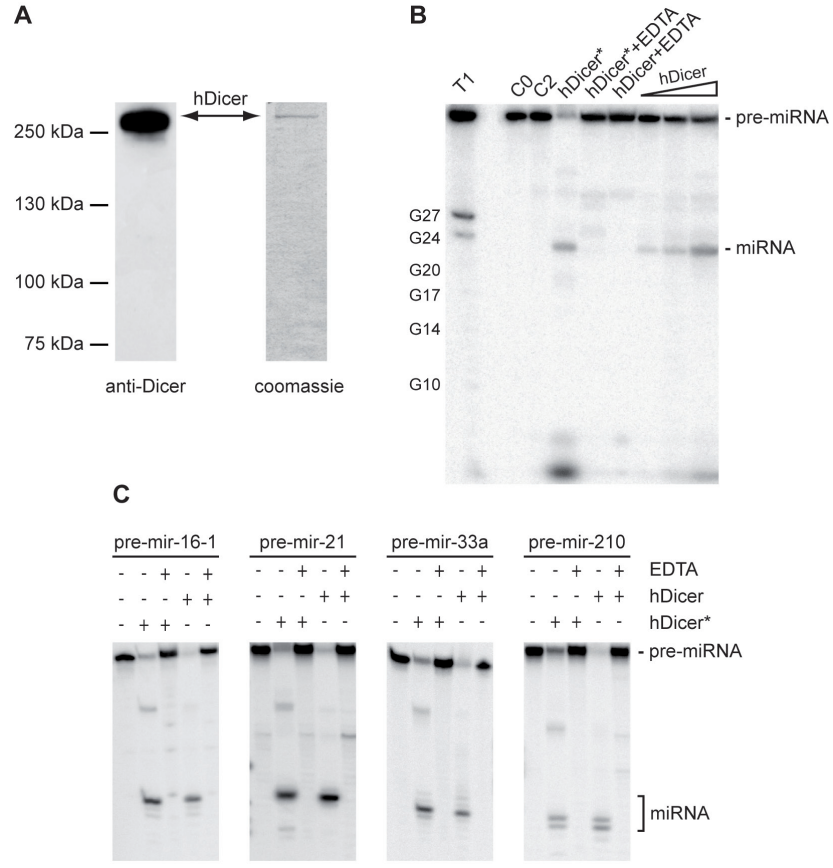

Figure 1. Characterization of the two-step purified hDicer.
(A) PAGE analysis of the hDicer preparation. The polyhistidine-tagged hDicer protein was expressed in the Baculovirus Expression System, purified by Ni^{2+} affinity chromatography followed by ion exchange chromatography, and analyzed by SDS–PAGE followed by Western blotting with anti-Dicer antibody (*left*), or Coomassie Blue staining (*right*). The band corresponding to hDicer is indicated. **(B)** The effect of increasing hDicer concentration on miRNA formation. The ^{32}P-labeled pre-mir-21 was incubated with increasing amounts of the tested hDicer preparation (1, 2.5 and 5 µl; represented by *a triangle*) or with a commercially available hDicer (*hDicer**, 1U), and analyzed by denaturing PAGE. *C0* and *C2* – controls incubated without hDicer for 0h and 2h, respectively; *hDicer*/hDicer+EDTA* – a reaction carried out with either a commercial enzyme or hDicer, in a buffer supplemented with 50 mM EDTA; *T1* – G-ladder generated with RNase T1. **(C)** Comparison of the hDicer cleavage activity on different pre-miRNAs. The ^{32}P-labeled pre-miRNA, as indicated, was incubated with the tested hDicer preparation or with a commercially available enzyme (*hDicer**). The presence of hDicer/hDicer* and supplementation of the reaction buffer with 50 mM EDTA are indicated with (+).

RESULTS AND DISCUSSION

Influence of the homogeneity of the recombinant human Dicer on its RNA-cleavage and RNA-binding properties

In 2002, two research groups independently reported in the back-to-back articles the production and characterization of hDicer (Provost *et al.*, 2002; Zhang *et al.*, 2002). Both groups

used the Baculovirus Expression System to obtain hDicer in insect cells. Since then, this enzyme has been successfully produced by many research groups (Ma *et al.*, 2008; Lima *et al.*, 2009). Preparations of hDicer are also commercially available. Our previous studies focusing on the regulation of the hDicer cleavage activity by short RNA molecules were carried out with commercial enzymes (Tyczewska *et al.*, 2011; Kurzynska-Kokorniak *et al.*, 2013). While we found them appropriate for assays based on pre-miRNA cleavage reactions, they do not seem to be suitable for detailed RNA-binding studies. Under the applied conditions, the commercial hDicer preparations that we used did not form stable complexes with pre-miRNAs. Therefore, we established our own system for the production of hDicer in insect cells (for details see Materials and Methods). Based on the procedure reported by Provost *et al.*, we initially applied a one-step purification strategy involving Ni^{2+} affinity chromatography (Provost *et al.*, 2002). The hDicer preparation obtained (Supplementary Fig. S1A) was assayed for its RNase activity. We carried out two sets of cleavage assays. In the first set, ^{32}P-labeled pre-mir-21 was incubated with increasing amounts of the hDicer preparation (1, 2.5 and 5 μl) for 2 h at 37°C. Two control reactions without hDicer were also carried out to test the integrity of the substrate before the reaction was started and after it was completed. To verify whether the observed cleavage was the result of the Mg^{2+}-dependent RNase activity of hDicer, an additional control reaction contained the hDicer preparation and 50 mM EDTA. To assess specificity and efficiency of the preparation obtained, a reaction with a commercially available hDicer was carried out. The second set of reactions involved time-dependent assays. Reaction mixtures contained 5 μl of hDicer and ^{32}P-labeled pre-mir-21, and were incubated for 10 min, 30 min, 1.5 h, 3 h and 5 h at 37°C. All reaction mixtures were separated by denaturing PAGE and visualized by phosphorimaging. In general, the data collected indicated that the hDicer preparation after one-step purification by Ni^{2+} affinity chromatography displayed the RNase activity and generated the specific miRNA products. However, an increase in the hDicer concentration (Supplementary Fig. S1B at www.actabp.pl), and/or incubation for longer than 30 min (Supplementary Fig. S1B, C at www.actabp.pl), caused the level of miRNA product to be decreased. Because in all of these reactions we also observed a rapid decrease in the substrate, we concluded that the tested hDicer preparation contained some factor/s that might degrade both, the substrate and the product. Alternatively, the enzyme processed pre-miRNAs very efficiently, but resultant miRNAs were further digested by an as yet unknown factor/s. Addition of the commercial RNase-inhibitor cocktails did not significantly reduce degradation of the substrates and products (data not shown). We further assayed the hDicer preparation for the RNA-binding capacity. To this end, ^{32}P-labeled pre-mir-21 was incubated with increasing amounts of the hDicer preparation. Reaction mixtures were separated by native PAGE and visualized by phosphorimaging (Supplementary Fig. S1D at www.actabp.pl). We found that in addition to a band corresponding to a putative hDicer-pre-miRNA complex, smeared bands and well-complexes (material that did not migrate out of the wells) were also formed. The comparison of the homogeneity of the obtained hDicer preparation and commercial enzymes showed that they were purified to a similar level (see Supplementary Fig. S1A and Supplementary Fig. S2 at www.actabp.pl). We repeated the RNA-cleavage and RNA-binding assays with different batches of the one-step purified hDicer; however, we did not obtain reproducible results. Thus, the hDicer preparation was subjected to further purification involving ion-exchange chromatography. The two-step purified hDicer (Fig. 1A) was tested for its RNase and RNA-binding capacities, according to the above protocols. Results of these assays are shown in Fig. 1B. The data collected revealed that an increase in hDicer concentration was accompanied by an increase in the miRNA product. In the next experiment, we evaluated the RNase activity of the two-step purified hDicer using different pre-miRNA substrates; namely, pre-mir-16-1, -21, -33a and -210. In each reaction set, a ^{32}P-labeled pre-miRNA was incubated with 5 μl of hDicer for 2 h at 37°C. Control reactions were carried out with a commercial enzyme. Another two control reactions contained either a commercial hDicer or our two-step purified hDicer preparation, and 50 mM EDTA (Fig. 1C). These experiments indicated that the obtained preparation processed pre-mir-16-1, -21, 33a and -210 with almost equal efficiencies. We also found that the efficiency of miRNA production by our preparation was comparable to the efficiency of miRNA production by commercial enzymes. Finally, to evaluate the amount of the enzyme optimal for the RNA cleavage assays, ^{32}P-labeled pre-mir-21 was incubated with increasing amounts of hDicer (20–650 nM) for 2 h at 37°C. Protein concentration was estimated by using the Bradford reagent, with BSA as a standard. Based on results obtained from three independent experiments, we calculated the cleavage efficacy (the ratios between the substrate (pre-mir-21) and the product

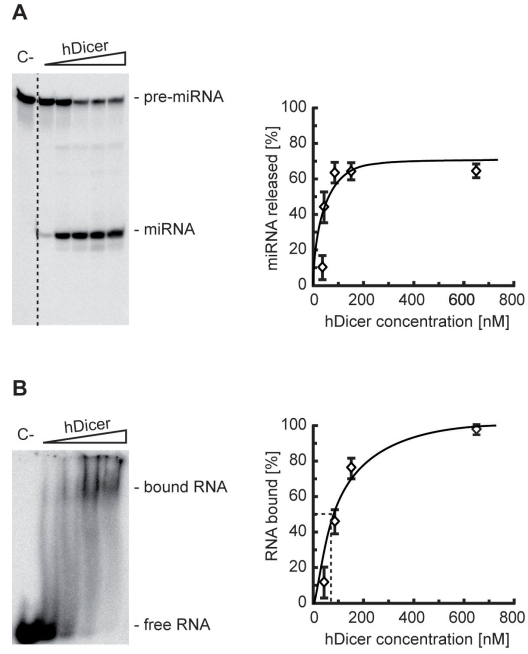

Figure 2. RNA-cleavage and RNA-binding properties of the two-step purified hDicer.
(A) Quantitative analysis of pre-miRNA processing by hDicer. The ^{32}P-labeled pre-mir-21 was incubated in the absence (*C-*) or presence of hDicer (20, 40, 80, 160, 650 nM; represented by *a triangle*) and analyzed by denaturing PAGE. The fraction of miRNA released by hDicer was determined and fit as a function of the protein concentration. Error bars represent standard deviation of three independent experiments. **(B)** Determination of the equilibrium dissociation constant of the hDicer and pre-miRNA complex. The ^{32}P-labeled pre-mir-21 was incubated in the absence (*C-*) or presence of hDicer (20, 40, 80, 160, 650 nM; represented by *a triangle*) and analyzed by EMSA. The fraction of bound RNA was determined and fit as a function of hDicer concentration. Error bars represent standard deviation of three independent experiments.

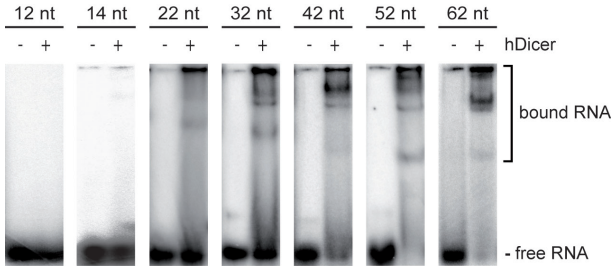

Figure 3. Binding between hDicer and single-stranded RNAs of different length.
The ssRNA-binding capacity of hDicer was tested by EMSA. The ^{32}P-labeled oligonucleotides (RNA12, RNA22, RNA32, RNA42, RNA52, RNA62) were incubated in the absence or presence of hDicer, denoted (-) and (+), respectively. The length of the tested RNAs is given above each panel.

(miR-21)) for each reaction. The average percentage of the product released was plotted against the molar concentrations of hDicer. We found that the maximum efficacy of the substrate cleavage was achieved at a hDicer concentration of ~150 nM (Fig. 2A). In addition, we attempted to determine the K_d value for the hDicer and pre-miRNA complex using EMSA. As shown in Fig. 2B, the K_d value for the hDicer and pre-mir-21 complex was 80±5.6 nM, which is of the same order of magnitude as previously reported for hDicer and other pre-miRNAs (Ma *et al.*, 2008; Feng *et al.*, 2012).

Binding between hDicer and single-stranded RNAs of different length

Our previous studies showed that the activity of hDicer can be affected by short RNA molecules binding to the enzyme, substrate or both (Tyczewska *et al.*, 2011; Kurzynska-Kokorniak *et al.*, 2013; Kurzynska-Kokorniak *et al.*, 2015). In the pool of the investigated ssRNAs there were species ranging from 12- to 62-nt. To explore which of the tested ssRNAs are able to interact with hDicer, we carried out binding assays involving 12-, 14-, 22-, 32-, 42-, 52- and 62-nt-long ssRNAs. The ^{32}P-labeled ssRNAs were incubated with or without hDicer for 30 min at 4°C and analyzed by native PAGE. The results, shown in Fig. 3, demonstrated that hDicer efficiently bound ssRNAs longer than ~20-nt, while it did not form a complex with a 12-nt ssRNA, and binding of a 14-nt ssRNA was very inefficient. These results also confirmed our previous observations that 12-nt ssRNA oligonucleotides, designed to interact with apical and/or single-stranded regions of pre-miRNAs, do not interact with hDicer (Kurzynska-Kokorniak *et al.*, 2013).

Very recently, we showed that the DUF283 domain of hDicer can bind 12-nt ssRNA *in vitro*, though the observed binding was very weak (Kurzynska-Kokorniak *et al.*, 2016). Here, we demonstrated that the full length hDicer does not bind such short ssRNAs (Fig. 3). Nevertheless, in the case of the separate DUF283 domain, less than half of 12-nt ssRNA was bound at DUF283 concentration of ~8.0 μM (Kurzynska-Kokorniak *et al.*, 2016). Our *in vitro* binding, cleavage and inhibition assays involved hDicer at concentrations in the range from 20 to 650 nM. Thus, we concluded that under such conditions, complexes between 12-nt ssRNA and hDicer were not formed or they were formed so inefficiently that we were not able to detect them. In general, the data collected suggest that binding of 12-14-nt ssRNAs by hDicer, if possible, occurs highly inefficiently. hDicer can effectively bind ssRNAs longer than ~20-nt; the longer ssRNA, the more efficient binding between hDicer and ssRNA was observed.

Regulation of pre-miRNA processing by siRNA/miRNA-sized single stranded RNAs

None of our earlier inhibition assays involved siRNA/miRNA-sized RNA oligonucleotides. As shown in Fig. 3 and demonstrated by Kini and Walton (Kini & Walton, 2007), and Lima *et al.* (Lima *et al.*, 2009), ~20-nt ssRNAs can bind to hDicer, although this binding is generally inefficient. Thus, it is possible that siRNA/miRNA-sized ssRNAs could inhibit, to some extent, the activity of hDicer by acting as competitive or allosteric regulators. Interestingly, in 2012 Pasquinelli and coworkers characterized an auto-regulatory loop involving let-7 miRNA (Zisoulis *et al.*, 2012). The authors have found that in *Caenorhabditis elegans,* mature let-7 miRNA may target a complementary site at the 3' end of the let-7 primary

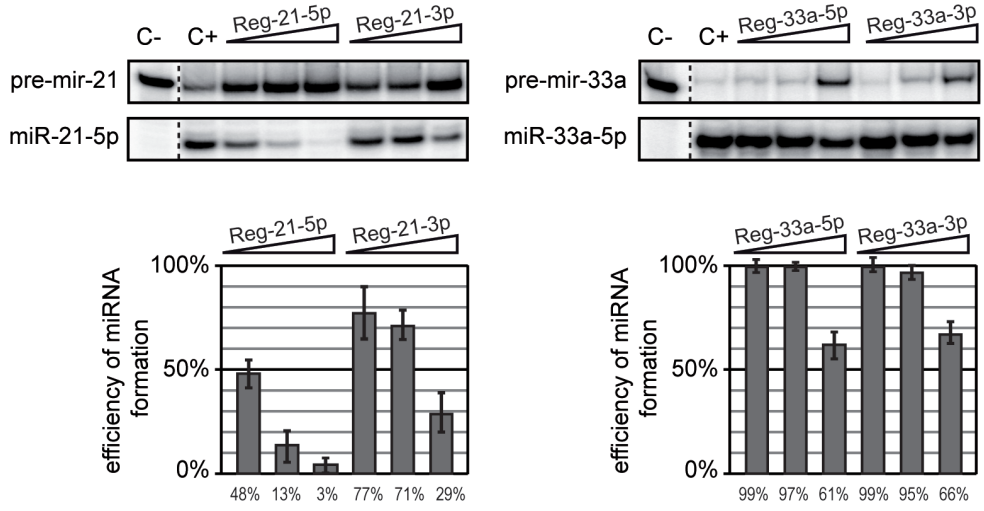

Figure 4. Influence of ~20-nt miR-regulators on hDicer activity.
The ^{32}P-labeled pre-miRNA (pre-mir-21 or pre-mir-33a) was incubated with hDicer in the presence of the indicated miR-regulator, and analyzed by denaturing PAGE. Control reactions lacked the enzyme and miR-regulator (C-) or miR-regulator only (C+). *Triangles* represent increasing amounts of a miR-regulator (pre-miRNA and miR-regulator molar ratios of 1:1, 1:10, and 1:100). Diagrams show the average efficiency of miRNA production in comparison to C+; error bars represent the standard deviation from three independent experiments.

transcripts, in this way promoting their processing. Considering all the above information, we asked the question whether siRNA/miRNA-sized ssRNAs might selectively influence production of the individual miRNAs by hDicer. To answer this problem, the hDicer cleavage assays involving either pre-mir-21 or pre-mir-33a were performed. Each of these precursors gives rise to two functional miRNAs (miRNA-5p and miRNA-3p), whose biological functions have been already well characterized (Doberstein *et al*., 2014; Pink *et al*., 2015). In the assays performed, we focused on production of miRNA-5p. As putative regulators of either pre-mir-21 or pre-mir-33a cleavage by hDicer, we used oligonucleotides identical with miRNAs (miRNA-5p and miRNA-3p), originating from these two precursors. We named them accordingly: Reg-5p and Reg-3p, while both were called miR-regulators. Reaction mixtures contained fixed concentrations of the 5' end-labeled pre-miRNA and of hDicer, and only the amount of the miR-regulators was altered as follows: 1:1, 1:10, 1:100, molar ratios of pre-miRNA to either Reg-5p or Reg-3p. In addition, two control reactions were always run, a reaction without hDicer (C-) and a reaction without a miR-regulator (C+). To allow interaction among pre-miRNA, miR-regulator and hDicer before cleavage, all mixtures were pre-incubated for 15 min in a buffer lacking Mg^{2+} ions. After that time, Mg^{2+} ions were added to induce hDicer cleavage and all samples were further incubated for 2h at 37°C. The amount of the pre-miRNA and miRNA-5p was determined for each reaction and the efficiency of miRNA-5p production in the presence or absence of Reg-5p/Reg-3p was calculated. The influence of a miR-regulator on miRNA production was expressed as a percentage, with 100% defined as the miRNA-5p production in reactions conducted without a miR-regulator. The collected results are presented in Fig. 4. In general, we found that all miR-regulators, at the highest concentrations used, affected the corresponding miRNA-5p production. In the case of the pre-mir-21 cleavage assay, we noticed that Reg-21-5p influenced formation of miR-21-5p more efficiently than did Reg-21-3p. Even at the lowest concentration used, Reg-21-5p reduced miR-21-5p production by ~50%. At the highest concentration used, Reg-21-5p almost completely repressed pre-mir-21 processing by hDicer, while at the same concentration, Reg-21-3p decreased production of miR-21-5p by ~70% (Fig. 4, left panel). In the case of the pre-mir-33a cleavage assay, we did not observe a significant difference between inhibition potency of Reg-33a-5p and Reg-33a-3p; both regulators at low concentrations did not affect cleavage of pre-mir-33a. At the highest concentration used, both reduced miR-33a-5p production by ~40% (Fig. 4, right panel). To test the specificity of the regulators used, we prepared control reactions that contained pairs: pre-mir-33a:Reg-21-3p and pre-mir-21:Reg-33a-3p, at pre-miRNA to Reg-3p molar ratios of 1:1 and 1:100 (Fig. 5A). We found that Reg-21-3p, even at the highest concentration used, did not affect pre-mir-33a cleavage by hDicer. In contrast, Reg-33a-3p, at its highest concentration, reduced miR-21-5p formation by ~90%. Providing the fact that at the same concentration Reg-33a-3p decreased miR-33a-5p production by ~40%, it was an unexpected result. Based on our previous observations, summarized in (Kurzynska-Kokorniak *et al*., 2013), we hypothesized that Reg-33a-3p interacted with pre-mir-21. Indeed, a secondary structure predicted by the RNAstructure program, revealed that Reg-33a-3p could bind to pre-mir-21 (Fig. 5B), which was further confirmed by EMSA (data not shown). Interestingly, results of our experiments are in line with the observations of Tang et *al*., who demonstrated that one miRNA can control the biogenesis of other miRNAs by base-pairing with their precursors (Tang *et al*., 2011). The authors showed that in mouse, miR-709 directly binds to a 19-nt

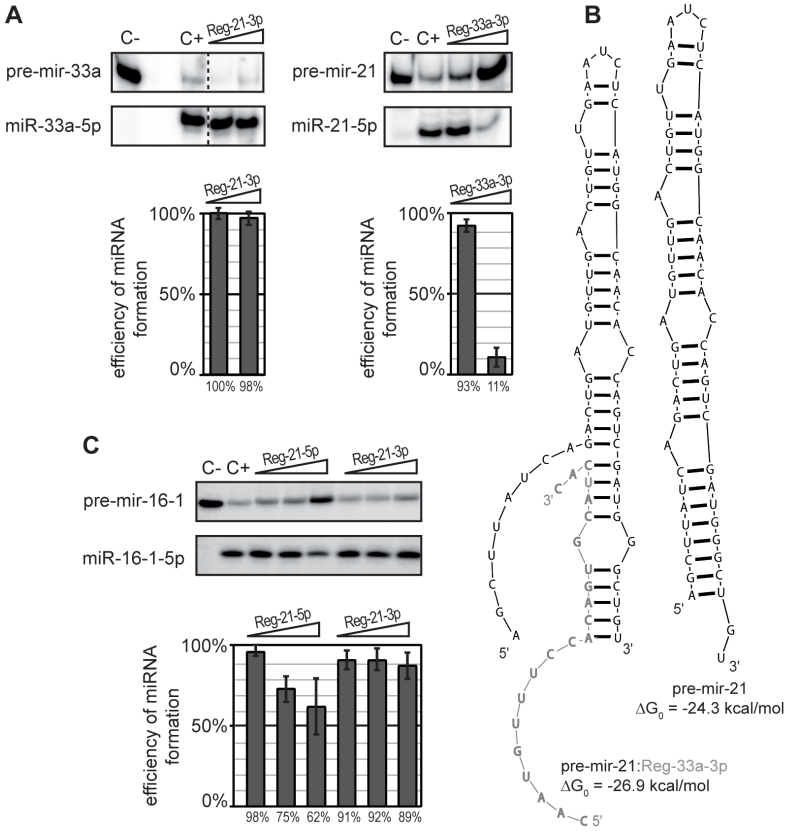

Figure 5. Analysis of the specificity of miR-regulators.
(A) The influence of non-matching miR-regulators on pre-miRNA processing by hDicer. The ^{32}P-labeled pre-miRNA (pre-mir-21 or pre-mir-33a) was incubated with hDicer and the non-matching miR-regulator. Control reactions lacked the enzyme and miR-regulator (*C*-) or miR-regulator only (*C*+). *Triangles* represent increasing amounts of miR-regulator (pre-miRNA and miR-regulator molar ratios of 1:1, and 1:100). Diagrams show the average efficiency of miRNA production in comparison to *C*+; error bars represent standard deviation of three independent experiments. **(B)** *In silico* prediction of the pre-mir-21 and Reg-33a-3p complex secondary structure.
(C) The influence of Reg-21 on pre-mir-16-1 processing by hDicer. ^{32}P-labeled pre-mir-16-1 was incubated with hDicer in the presence of the indicated miR-regulator (either Reg-21-5p or Reg-21-3p), and analyzed by denaturing PAGE. Control reactions lacked the enzyme and miR-regulator (*C*-) or miR-regulator only (*C*+). *Triangles* represent increasing amounts of the miR-regulator (pre-miRNA and miR-regulator molar ratios of 1:1, 1:10 and 1:100). Diagrams show the average efficiency of miRNA production in comparison to *C*+; error bars represent standard deviation of three independent experiments.

miR-709 recognition element on primary transcript pri-miR-15a/16-1, in this way preventing its processing into pre-mir-15a/16-1 and in consequence preventing formation of mature miR-15a/16-1.

Data shown in Fig. 4 and Fig. 5A, B suggested that some miRNAs might act as specific regulators; as is the case of Reg-21-3p. To test whether Reg-21-3p might be a specific regulator, we performed another set of control experiments involving pre-mir-16-1 and either Reg-21-5p or Reg-21-3p (Fig. 5C). The obtained results revealed that Reg-21-5p, at the highest concentration used, inhibited miR-16-1 production by ~40%, though both molecules do not share complementary sequences. At the same concentrations, Reg-21-3p only slightly influenced miR-16-1 production. These results further confirmed that Reg-21-3p might act as a specific inhibitor of miR-21-5p production. Very recently, we showed that hDicer and its DUF283 domain can accelerate annealing of complementary ssRNAs (Kurzynska-Kokorniak *et al.*, 2016). Given that all cleavage reactions were preceded by 15 min incubation with hDicer and they included complementary RNAs (pre-miRNA and the respective miR-regulator), we assumed that the characterized phenomenon might also apply to the performed pre-miRNA cleavage assays. Therefore, we monitored the effect of pre-incubation with hDicer, in the absence of Mg^{2+} ions, for pairs: pre-mir-21 and Reg-21-5p, pre-mir-21 and Reg-21-3p, pre-mir-33a and Reg-33a-5p, pre-mir-33a and Reg-33a-3p, at 1:100 molar ratio of pre-miRNA to miR-regulator. The results obtained are presented in Supplementary Fig. S3 (at www.actabp.pl). We found that Reg-21-3p efficiently base-paired with pre-mir-21, and that reaction was strongly accelerated by hDicer. Thus, these results suggested that Reg-21-3p might act as an in-cis-regulator of miR-21 production by binding with its precursor. Our preliminary *in vitro* experiments were restricted to a few miRNA cases. Thus, there is no doubt that more extensive *in vitro* and in cell culture studies are needed to confirm that an auto-regulatory loop between miRNA and pre-miRNA does exist.

We also found that in the case of the pair: pre-mir-21 and Reg-21-5p, the hDicer-assisted annealing was very inefficient (Supplementary Fig. S3). Likewise, we found that Reg-21-5p bound to hDicer with similar efficiency as other ~20-nt ssRNAs tested (Fig. 3 and data not shown). Thus, at present we cannot explain the mechanism underlying such high inhibitory potential of Reg-21-5p and apparently more detailed studies are needed to elucidate the observed phenomenon. Finally, annealing was not detected for pairs: pre-mir-33a and Reg-33a-5p, pre-mir-33a and Reg-33a-3p (Supplementary Fig. S3 at www.actabp.pl). In addition, inhibition assays presented in Fig. 4 indicated that Reg-33a-5p/ -3p affected miR-33a-5p production only to a small degree. Thus, we deduced that the observed inhibition resulted from weak binding of Reg-33a-5p/ -3p to hDicer (Fig. 3; lane marked [22 nt] represents the results of EMSA containing Reg-33a-3p and hDicer).

In conclusion, we postulate that all tested ~20-nt ssRNAs can, to some extent, interact with hDicer, thereby sequestering its cleavage activity. It has been reported that the efficiency of ~20-nt ssRNAs binding to Dicer strongly depends on their sequence (Lima *et al.*, 2009). These observations of Lima *et al.* might, at least in part, explain different inhibitory potential presented by the four ~20-nt miR-regulators used, each having a different RNA sequence. In addition, we found that two miR-regulators, one identical with miR-21-3p and the other identical with miR-33a-3p, could act as an in-cis- and in-trans-inhibitor, respectively, of pre-mir-21 cleavage by hDicer.

Different mechanisms of the hDicer activity regulation by short single-stranded RNAs

Based on the data collected during our earlier and present studies, we can classify the mechanisms by which RNA oligonucleotides affect hDicer activity *in vitro* into two groups: (i) the mechanisms involving direct binding of oligonucleotides to hDicer and (ii) the mechanisms based on oligonucleotide interactions with hDicer substrates. i.e., pre-miRNAs (Tyczewska *et al.*, 2011; Kurzynska-Kokorniak *et al.*, 2013). The first group encompasses the *in vitro* selected RNA oligonucleotides (Tyczewska *et al.*, 2011). Although these oligonucleotides form a relatively homogenous group of RNAs in terms of their length (42-62-nt ssRNAs, mostly 56-nt) and the structure (stem-loop), we found that they differ in their potential to inhibit pre-miRNA cleavage by hDicer due to the different affinities to this enzyme. Depending on whether they are cut by hDicer or not, we can classify them as competitive or allosteric inhibitors, respectively (Fig. 6, I). The second group involves RNAs that are capable of base-pairing with pre-miRNAs (Kurzynska-Kokorniak *et al.*, 2013). As a consequence of interactions between the oligonucleotide and the pre-miRNA, the native structure of the latter is disturbed. The resultant complex of RNA oligonucleotide and pre-miRNA either is not recognized and digested by hDicer (Fig. 6, II and 6, IIIa) or, when recognized and processed by hDicer, the pattern of pre-miRNA cleavage is altered and mature, functional miRNAs are not formed (Fig. 6, IIIb). The scenario presented in Fig. 6, II takes into consideration oligonucleotides that are too short to bind to hDicer (RNAs shorter than 20-nt, as shown in Fig. 3). However, they can selectively base-pair with single-stranded regions of the individual pre-miRNAs and act as specific inhibitors that preclude the formation of the corresponding miRNAs.

Some of the tested oligonucleotides could be assigned to both groups, thus we named them bifunctional inhibitors. Bifunctional inhibitors are recognized and bound by hDicer but they also contain sequences complementary to pre-miRNAs. We characterized several such inhibitors, including 56-nt long ATD_15.52, ATD_13.6 (Kurzynska-Kokorniak *et al.*, 2013), ATD_15.2 (Supplementary Fig. S4 at www.actabp.pl) and ~30-nt long PCDH21_fr, and THAP4_fr (Supplementary Fig. S5). In the case of ATD_15.52, we found that this oligonucleotide competes with pre-mir-210 for binding to hDicer; thus it acts as a competitive inhibitor. After ATD_15.52 cutting by hDicer, its 5' fragment binds to the apical region of pre-mir-210 due to complementarity between their sequences and inhibits pre-miRNA processing (the scenario presented in Fig. 6, Ia). ATD_13.6 is also bound by hDicer, but it is not processed by this enzyme; thus we hypothesize that it acts as an allosteric inhibitor. In addition, ATD_13.6 base-pairs with pre-mir-210 and completely disturbs the native structure of the precursor. We found that the ATD_13.6 and pre-mir-210 complex is not processed by hDicer (the scenario presented in Fig. 6, IIIa) (Kurzynska-Kokorniak *et al.*, 2013). Interestingly, we also identified in our collection an oligonucleotide named ATD_15.2 that hybridizes with pre-mir-210, and the resultant dimer is recognized and processed by hDicer. In this case, however, the pattern of pre-mir-210 cleavage is altered and the functional miR-210 is not produced (the scenario presented in Fig. 6, IIIb) (Sup-

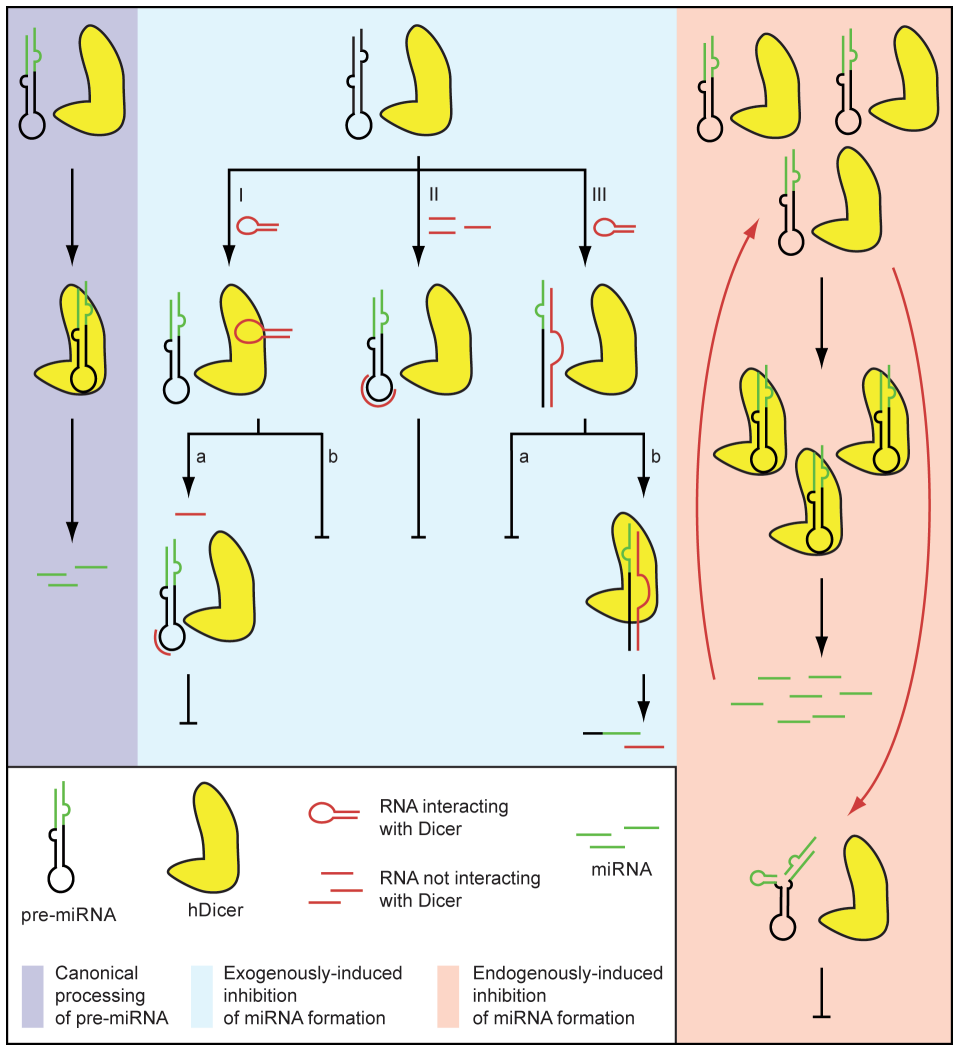

Figure 6. Proposed scenarios of short RNA-based regulation of hDicer cleavage activity.
A canonical pathway of pre-miRNA processing by hDicer (*left*) can be interfered by RNA oligonucleotides of various length. Oligonucleotides may affect hDicer activity by interacting with the enzyme (*I*) or pre-miRNA (*II, III*), thus inhibiting miRNA formation (*I, II, IIIa*) or altering the substrate cleavage pattern (*IIIb*). A possible mechanism of an auto-regulatory feedback loop (*right*).

plementary Fig. S4 at www.actabp.pl). In the set of bifunctional inhibitors there are also two ~30-nt ssRNAs identical with fragments of some transcripts, accordingly: PCDH21_fr, a 30-nt fragment of PCDH21 (*H. sapiens* protocadherin 21, potential calcium-dependent cell-adhesion protein) mRNA and THAP4_fr, a 35-nt fragment of THAP4 (*H. sapiens* THAP domain containing 4 protein, i.e., DNA and metal ion binding protein) mRNA (Tyczewska *et al.*, 2011). Both oligonucleotides contain sequences complementary to pre-miR-210, but they can also interact with hDicer (Fig. 3). Inhibition assays carried out with pre-miR-210 and either PCDH21_fr or THAP4_fr are presented in Supplementary Fig. S5 (at www.actabp.pl), and the possible mechanism of inhibition displayed by these two oligonucleotides matches the scenario demonstrated in Fig. 6, IIIa.

Finally, we hypothesize that miRNAs that are produced at a high level, thus reaching a high local concentration, might interact with pre-miRNAs from which they derive (Fig. 6, right panel). Our observations are consistent with the earlier mentioned observations published by Pasquinelli *et al.*, who have reported that let-7 miRNA maturation can be controlled by an auto-regulatory loop between the let-7 miRNA and let-7 pri-miRNA (Zisoulis *et al.*, 2012). Alternatively, miRNAs produced might target complementary pre-miRNAs other than their own precursors and, in this way, *in trans* repress their cleavage by Dicer (Fig. 5). Accordingly, Tang et *al.* have demonstrated that miR-709 can base-pair with pri-miR-15a/16-1, thereby preventing its processing into pre-mir-15a/16-1 (Tang *et al.*, 2011).

All data presented in this manuscript were obtained *in vitro* and obviously it is not clear whether similar mechanisms of the Dicer activity regulation might exist in cells. Thus, the problem of RNA-based regulation of Dicer needs to be further explored *in vivo*. Nevertheless, growing evidence shows that the cytoplasm contains a wide spectrum of RNA molecules that hypothetically might interfere with miRNA biogenesis pathways (Jackowiak *et al.*, 2011b; Nowacka *et al.*, 2012; Kurzynska-Kokorniak *et al.*, 2015).

It is now clear that one of the most challenging issues of the biomedical field is designing and production of therapeutics that selectively interact with specific targets. Our data indicate that one can modulate the production of individual miRNAs by using specific RNA oligonucleotides. The application of such RNA molecules might be useful for the treatment of many diseases associated with the aberrant regulation of specific miRNA levels, like cancers, neurodegenerative diseases, and even infec-

tious diseases caused by such dangerous viruses as HIV or HCV (Figlerowicz *et al*., 2003; Kurzynska-Kokorniak *et al*., 2009; Miazga *et al*., 2011; Jackowiak *et al*., 2012; Gorska *et al*., 2013; Jackowiak *et al*., 2014; Dutkiewicz *et al*., 2015; Belter *et al*., 2016).

Acknowledgements

This work was supported by the European Union Regional Development Fund within the PARENT-BRIDGE Program of the Foundation for Polish Science [Pomost/2011-3/5 to A.K.K.]. This publication was also supported by the National Science Centre [Maestro 2012/04/A/NZ1/00056 to K.B.S.] and by the Polish Ministry of Science and Higher Education under the KNOW program.

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