Cellular Localization and Processing of Primary Transcripts of Exonic MicroRNAs

Slezak-Prochazka, Izabella; Kluiver, Joost; de Jong, Debora; Kortman, Gertrud; Halsema, Nancy; Poppema, Sibrandes; Kroesen, Bart J.; van den Berg, A.

Published in:
PLoS ONE

DOI:
10.1371/journal.pone.0076647

IMPORTANT NOTE: You are advised to consult the publisher's version (publisher's PDF) if you wish to cite from it. Please check the document version below.

Document Version
Publisher's PDF, also known as Version of record

Publication date:
2013

Link to publication in University of Groningen/UMCG research database

Citation for published version (APA):
Slezak-Prochazka, I., Kluiver, J., de Jong, D., Kortman, G., Halsema, N., Poppema, S., ... van den Berg, A. (2013). Cellular Localization and Processing of Primary Transcripts of Exonic MicroRNAs. PLoS ONE, 8(9), [e76647]. DOI: 10.1371/journal.pone.0076647

Copyright
Other than for strictly personal use, it is not permitted to download or to forward/distribute the text or part of it without the consent of the author(s) and/or copyright holder(s), unless the work is under an open content license (like Creative Commons).

Take-down policy
If you believe that this document breaches copyright please contact us providing details, and we will remove access to the work immediately and investigate your claim.

Downloaded from the University of Groningen/UMCG research database (Pure): http://www.rug.nl/research/portal. For technical reasons the number of authors shown on this cover page is limited to 10 maximum.
Cellular Localization and Processing of Primary Transcripts of Exonic MicroRNAs

Izabella Slezak-Prochazka, Joost Kluiver, Debora de Jong, Gertrud Kortman, Nancy Halsema, Sibrand Poppema, Bart-Jan Kroesen, Anke van den Berg

Department of Pathology and Medical Biology, University Medical Center Groningen, University of Groningen, Groningen, The Netherlands

Abstract

Processing of miRNAs occurs simultaneous with the transcription and splicing of their primary transcripts. For the small subset of exonic miRNAs it is unclear if the unspliced and/or spliced transcripts are used for miRNA biogenesis. We assessed endogenous levels and cellular location of primary transcripts of three exonic miRNAs. The ratio between unspliced and spliced transcripts varied markedly, i.e. >1 for BIC, <1 for pri-miR-146a and variable for pri-miR-22. Endogenous unspliced transcripts were located almost exclusively in the nucleus and thus available for miRNA processing for all three miRNAs. Endogenous spliced pri-miRNA transcripts were present both in the nucleus and in the cytoplasm and thus only partly available for miRNA processing. Overexpression of constructs containing the 5’ upstream exonic or intronic sequence flanking pre-miR-155 resulted in strongly enhanced miR-155 levels, indicating that the flanking sequence does not affect processing efficiency. Exogenously overexpressed full-length spliced BIC transcripts were present both in the nucleus and in the cytoplasm, were bound by the Microprocessor complex and resulted in enhanced miR-155 levels. We conclude that both unspliced and spliced transcripts of exonic miRNAs can be used for pre-miRNA cleavage. Splicing and cytoplasmic transport of spliced transcripts may present a mechanism to regulate levels of exonic microRNAs.


Received August 23, 2012; Accepted August 30, 2013; Published September 20, 2013

Copyright: © 2013 Slezak-Prochazka et al. This is an open-access article distributed under the terms of the Creative Commons Attribution License, which permits unrestricted use, distribution, and reproduction in any medium, provided the original author and source are credited.

Funding: This study was supported by a grant from the Dutch Cancer Society (RUG 2009-4279) to B.J.K. and A.v.d.B and by a grant from the Ubbo Emmius Foundation. The funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript.

Competing interests: The authors have declared that no competing interests exist.

* E-mail: a.van.den.berg01@umcg.nl

Introduction

MicroRNAs (miRNAs) are small (~22nt) noncoding RNA molecules that negatively regulate gene expression by binding to the 3’ untranslated region (3’ UTR) of their target mRNAs [1]. MiRNAs play an important role in cellular processes like apoptosis, proliferation and differentiation. Altered miRNA expression profiles have been associated with various diseases including most, if not all, types of cancer [2]. This suggests that regulation of miRNA levels is important for normal cellular functioning. Regulation of the miRNA levels may include any of the regulatory mechanisms involved in normal gene expression, such as transcriptional or epigenetic control of transcription. In addition to these transcriptional regulations, miRNA levels can also be regulated at the post-transcriptional level during miRNA processing (reviewed in 3).

The first step in miRNA processing, i.e. cleavage of the primary miRNA transcript (pri-miRNA) by the Drosha/DGCR8 complex, is restricted to the nucleus [4-7]. The miRNA stem-loop structures can be located in introns of protein-coding or noncoding RNA genes, in exons of noncoding genes or in intergenic regions [8]. The vast majority of the human miRNAs are located in introns. Approximately 10% of the miRNAs, including miR-155, miR-146a, miR-22, miR-137, miR-34c and let-7b, reside within exons of noncoding genes [9-11]. Current knowledge about processing of pri-miRNAs has been obtained mainly for intronic or intergenic miRNAs [10,12-16]. Processing of intronic miRNAs occurs co-transcriptionally in cooperation with splicing of the primary transcript [10,12,13,15,16]. The Microprocessor complex and the spliceosome are associated in one complex, and co-produce precursor miRNAs (pre-miRNA) and spliced transcripts from the unspliced pri-miRNA [15]. Splicing is not required for pri-miRNA processing [10], but spliceosome assembly may promote release of the pre-miRNA from introns of pri-miRNA [15]. For exonic miRNAs, pre-miRNA release will disrupt the exon of the pri-miRNA and affect formation of spliced transcripts. Therefore, it is more likely that unspliced pri-miRNA transcripts of exonic miRNAs produce either pre-miRNAs or spliced transcripts. The processing of exonic miRNAs has not yet been studied in detail.

One well-known exonic miRNA, miR-155, is processed from the transcript of the B-cell integration cluster (BIC) gene, also
known as the MIR155 host gene (MIR155HG) [17]. The BIC gene consists of three exons separated by long (7.6 and 4kb) introns and the stem-loop pre-miR-155 sequence is located in the third exon [18]. MiR-155 is crucial for B-cell development and regulation of the immune response [9,19,20]. High miR-155 levels are observed in many types of cancer, including B-cell malignancies like Hodgkin, primary mediastinal and diffuse large B-cell lymphomas [21,22]. In contrast, very low levels of miR-155 were observed in B cell-derived Burkitt lymphoma [23]. Eis et al. showed that unspliced BIC is located in the nucleus, whereas spliced BIC is located mainly in the cytoplasm in two B-cell lymphoma cell lines that both show high miR-155 levels [24]. RNA in situ hybridization in primary cases of Hodgkin lymphoma and non-Hodgkin lymphomas with high miR-155 levels revealed a strong nuclear staining of BIC and no staining in the cytoplasm [21,23].

In this study, we investigated processing of exonic miRNAs, with a main focus on miR-155. We determined the levels of endogenous unspliced and spliced BIC, pri-miR-22 and pri-miR-146a transcripts. We assessed cellular localization of endogenous unspliced and spliced BIC, pri-miR-22 and pri-miR-146a and showed that unspliced transcripts are located predominantly in the nucleus while spliced transcripts are partly transported to the cytoplasm. We also showed that the 5’ exonic or intronic flanking sequence of pre-miR-155 does not alter processing efficiency of exogenous BIC transcripts and that upon overexpression spliced BIC transcripts are efficiently processed to mature miR-155.

Results

The unspliced/spliced transcript ratio is miRNA-specific in B-cell lymphoma

For exonic miRNAs, such as miR-155, miR-22 and miR-146a, both unspliced and spliced transcripts include the complete stem-loop pre-miRNA sequence and may serve as the primary miRNA transcript. To discriminate between unspliced and spliced transcripts we designed qRT-PCR primer sets specific for unspliced or spliced transcripts as indicated in Figure 1A. We compared the levels of endogenous unspliced and spliced transcripts in twenty B-cell lymphoma cell lines with variable miRNA levels.

Endogenous miR-155 levels were highly variable in B-cell lymphoma cell lines. The difference between the cell line with the lowest (ST486) and the highest (OCI-Ly3) miR-155 level was ~500 fold (Figure S1A). The median level of the unspliced BIC transcript was 3.7 fold higher than that of the spliced BIC transcripts (Figure 1B). The levels of endogenous unspliced BIC transcripts were higher than the levels of spliced BIC transcripts in 17 out of 20 cell lines irrespective of the miR-155 levels (Figure S1B).

MiR-22 levels were low in all B-cell lymphoma cell lines (Figure S1C). Pri-miR-22 has four alternative splice variants. Transcript variant 3 was almost exclusively detected in our panel of B-cell lymphoma cell lines (data not shown). We therefore restricted our subsequent analysis to this splice variant. The medians of endogenous levels of unspliced and spliced pri-miR-22 were similar in 20 analyzed cell lines (Figure 1C). The spliced/unspliced pri-miR-22 transcript ratio varied from 0.2 to 8 (Figure S1D). In SU-DHL-4 cells, the unspliced pri-miR-22 transcript was hardly detected.

MiR-146a levels varied over a 1000-fold range between B-cell lymphoma cell lines (Figure S1E). The levels of spliced pri-miR-146a transcripts were significantly higher than unspliced pri-miR-146a transcripts with a more than 20 fold difference in medians (Figure 1D). The spliced/unspliced pri-miR-146a transcript ratio varied from 14 to 72 fold (Figure S1F). In L540 cells only the spliced pri-miR-146a transcript was present.

Next, we correlated the mature miRNA levels with either unspliced or spliced pri-miRNAs levels. For miR-155, both unspliced and spliced BIC transcript levels showed significant correlation with miR-155 levels (R² = 0.53). The difference observed in levels of unspliced and spliced BIC transcripts did not result in a significant difference in the slope of the regression lines. For miR-22, no significant correlation was observed for either spliced or unspliced pri-miR-22 transcript levels with mature miR-22 levels. This might be caused by differences in the efficiency of the pri-miR-22 transcript to miR-22 processing, differences in the miR-22 stability, factors regulating the levels of mature miR-22 or limiting amounts of factors involved in the biogenesis of miRNAs. For miR-146a, both unspliced and spliced pri-miR-146a transcript levels significantly correlated with miR-146a (R² = 0.67 and 0.7, respectively). However, the slopes of the curves differed significantly (p<0.0001) due to lower unspliced pri-miR-146a transcript levels.

Thus, for all three exonic miRNAs, both unspliced and spliced primary transcripts are present, albeit at a variable ratio. For BIC, the unspliced primary transcript is predominant, for pri-miR-146a the spliced primary transcripts is predominant, whereas for pri-miR-22 the unspliced/spliced transcript ratio varies between cell lines. Levels of the mature miRNAs correlated with levels of both unspliced and spliced pri-miRNA transcripts for miR-155 and miR-146a, but not for miR-22.

Spliced pri-miRNA transcripts are partly transported to the cytoplasm

To further examine, whether unspliced or spliced pri-miRNA transcripts are available for miRNA processing, we examined the subcellular localization of unspliced and spliced transcripts of BIC, pri-miR-22 and pri-miR-146a by qRT-PCR. We determined the amount of the spliced and unspliced transcripts in the cytoplasm and the nucleus relative to their levels in the total fraction (Figure 2). To asses subcellular localization of unspliced and spliced BIC transcripts, we selected cell lines with low (L428), intermediate (Jiyoye) or high (L540) miR-155 levels (Figure S1A). The same cell lines were used for subcellular localization of pri-miR-22 and pri-miR-146a to allow comparison between subcellular distributions of pri-miRNA transcripts. We confirmed purity of the nuclear and cytoplasmic fractions by analyzing relative abundance of tRNA-Lys in the cytoplasm, and U3 and Xist in the nucleus (Figure S2A-C).

Endogenous unspliced BIC transcripts were located exclusively in the nuclear fraction in all three cell lines (Figure 2A). Spliced BIC transcripts showed similar amounts in the nucleus and in the cytoplasm. Localization of unspliced pri-
miR-22 transcripts was predominantly, but not exclusively, nuclear in all three cell lines (Figure 2B). Spliced pri-miR-22 transcripts were present at similar levels in the nucleus and the cytoplasm in all three cell lines. Localization of unspliced pri-miR-146a transcripts was exclusively nuclear for both L428 and Jiyoye cells (Figure 2C). In L540 cells, both mature and primary miR-146a transcripts were hardly detected, so it was not possible to determine the subcellular localization. Spliced pri-miR-146a transcripts were more abundant in the nucleus than...
in the cytoplasm of L428 cells and in similar amounts in Jiyoye cells.

Thus, part of the spliced BIC, pri-miR-22 and pri-miR-146a transcripts are transported to the cytoplasm and as such unavailable for processing. Unspliced transcripts show an almost exclusive nuclear localization for BIC, pri-miR-22 and pri-miR-146a. Since the levels of spliced pri-miR-22 in L540 and Jiyoye cells and pri-miR-146a in Jiyoye and L428 were higher than the unspliced transcript levels (Figures S1D and S1F), spliced pri-miR-22 and pri-miR-146a may still be the predominant miRNA substrate.

**Exogenous spliced BIC can be processed to miR-155**

For further analysis we focused on miR-155, because previous studies have shown conflicting data concerning BIC to miR-155 processing [25,26]. The upstream pre-miR-155 flanking sequence is different in spliced and unspliced BIC transcripts. To determine if this upstream sequence affects the processing efficiency, we assessed the levels of miR-155 induction upon overexpression of BIC from two short fragments of BIC containing the stem-loop region, ~150nt 3’ flanking sequence from exon 3 and ~150nt 5’ flanking sequence derived either from intron 2 (s-intBIC) or from exon 2 (s-exBIC) of BIC transcript (Figure 1A). In addition, we also overexpressed the full-length spliced BIC (fl-exBIC) transcript (Figure 1A). We transduced these three BIC constructs into ST486, Ramos and U-HO1, i.e. cells that all have low endogenous miR-155 levels. A high expression of BIC was induced using either of the constructs (Figure 3A), albeit at variable levels. Transduction with the short exon spanning BIC construct resulted in the highest increase and the full-length spliced BIC construct resulted in the lowest increase in total BIC transcript levels (Figure 3A). Interestingly, the level of miR-155 induction was similar for all three constructs (Figure 3B), despite the marked differences in total BIC levels. These data indicate that the upstream pre-miR-155 flanking sequence does not modify processing efficiency and suggest that at a certain level of primary miRNA transcript, other factors become limiting or regulate the level of mature miR-155.

Next, we determined the subcellular localization of BIC transcripts in Ramos cells transduced with fl-exBIC (Figure 3C). Purity of cytoplasmic and nuclear fractions was validated with tRNA-Lys and U3, respectively (Fig. S2DE). In empty vector control cells, and in cells with overexpressed fl-exBIC, localization of endogenous unspliced BIC transcripts was predominantly nuclear. The endogenous spliced BIC transcripts were not detectable in empty vector control cells. The amount of the overexpressed fl-exBIC transcripts was similar in the cytoplasmic and nuclear fractions. Overexpression of fl-exBIC was followed by a strong induction of miR-155 (Figure 3B), indicating that upon overexpression nuclear spliced BIC transcripts can also serve as the primary miR-155 transcript.

To show that exogenous spliced BIC is processed to miR-155, we determined whether it is a direct target of the Microprocessor complex. We performed immunoprecipitation of DGCR8 [27] in wild-type Jiyoye, Ramos fl-exBIC and U-HO1 fl-exBIC cells (Figure 3D-F and Figure S3A-C). We assessed the relative amounts of unspliced and spliced BIC transcripts in the DGCR8 immunoprecipitation fraction (DGCR8-IP) compared to the control IgG-IP. Unspliced BIC transcript was significantly more enriched in DGCR8-IP fraction of all three cell lines, albeit at variable levels (Figure 3D). Similarly, the endogenous and exogenous spliced BIC transcripts were significantly enriched in DGCR8-IP fraction of Jiyoye, U-HO1 fl-exBIC and Ramos fl-exBIC cells (Figure 3E). The HPRT transcript used as negative
control was not enriched in the DGCR8-IP fractions (Figure 3F). Enrichment of endogenous and exogenous spliced BIC in the DGCR8-IP fractions indicate that the spliced primary transcript can be used for miR-155 processing.

The unspliced/spliced ratio of BIC transcripts changes upon cellular activation

To investigate whether the ratio between unspliced and spliced BIC transcripts is altered upon induction of BIC, we activated three B-cell lymphoma cell lines using PMA/Ionomycin. Activation of DG-75, L428 and KM-H2 cells resulted in a 3- to 13-fold increase in mir-155 levels (Figure 4A). Induction of unspliced BIC transcript levels showed a 1.6 to 5.5 fold increase, whereas spliced BIC transcript levels showed a 5.4 to 31 fold increase compared to untreated cells (Figure 4B).

Although the unspliced BIC transcript remained the predominant transcript, the unsploded/spliced BIC transcript ratio significantly changed in favour of the spliced BIC transcript (Figure 4C). Thus, both processing of unspliced BIC transcript to mir-155 and to spliced BIC transcripts are enhanced upon activation-induced expression of BIC. In addition, we also assessed induction of mature miR-146a and miR-22 as well as two intronic miRNAs (miR-191, miR-16) and one intergenic miRNA (let-7a). Induction of mature miRNA levels upon PMA/Ionomycin stimulation was observed only for the three exonic miRNAs (Figure S4). Both unspliced and spliced pri-miR-146a and pri-miR-22 transcript levels were

Figure 3. Processing and cellular localization of exogenous BIC transcripts. Levels of total BIC (A) and miR-155 (B) determined upon overexpression of the three constructs and empty vector (EV) in ST486, Ramos and U-HO1 cells. For the three BIC overexpression constructs, the increase in total BIC was variable and the highest levels were observed for the short BIC transcript containing the exon 2-derived 5’ pre-miR-155 flanking sequence (s-exBIC). However, miR-155 induction was similar for all BIC overexpression constructs. (C) The levels of spliced and unspliced BIC transcripts in nuclear and cytoplasmic fractions of Ramos EV and Ramos full-length spliced BIC (fl-exBIC). For both Ramos EV and fl-exBIC, unspliced BIC showed significantly higher (p<0.001) levels in the nucleus than in the cytoplasm. Spliced BIC was not detectable (ND) in Ramos EV. Upon overexpression of the fl-exBIC construct, spliced BIC transcripts showed similar levels in the cytoplasm and in the nucleus. Transcript levels in the cytoplasm and the nucleus were calculated relative to the total fraction and corrected for the amount of RNA per cell. Average of three independent experiments was presented. P values were determined by 2-way ANOVA and Bonferroni posttest (** p<0.01, ns - not significant). Relative amounts of unspliced (D) and spliced (E) BIC transcripts in DGCR8-IP compared to IgG-IP in Jiyoye, Ramos fl-exBIC and U-HO1 fl-exBIC cells. Endogenous unspliced, endogenous spliced and exogenous spliced BIC transcripts were significantly enriched in DGCR8-IP compared to IgG-IP. (F) No enrichment of HPRT transcript was observed in DGCR8-IP. Amounts were calculated relative to tRNA-Lys levels. P values were determined by Student’s t-test (** p<0.01, *** p<0.001, **** p<0.0001, ns - not significant).

doi: 10.1371/journal.pone.0076647.g003
induced upon PMA/Ionomycin treatment (Figure S4), however, no difference was observed for the unspliced/spliced transcripts ratios (Figure S4). Thus, the change in unspliced/spliced transcript ratio upon stimulation is specific for the BIC transcript.

Discussion

Exonic miRNAs constitute a small group of the known human miRNAs. The vast majority of exonic miRNAs are located in noncoding RNA genes of which the only known function is being the miRNA host gene. In contrast to intronic miRNAs, processing of the pri-miRNA transcripts of exonic miRNAs to pre-miRNAs interferes with the normal splicing process of the transcript. Exonic miRNAs regulate important physiological pathways, i.e. miR-155 and miR-146a are crucial regulatory components of the immune response, hematopoiesis and carcinogenesis (reviewed in 28,29) and miR-22 plays a role in carcinogenesis [30,31].

In this study, we showed that unspliced pri-miRNA transcripts of exonic miRNAs, i.e. miR-155, miR-22 and miR-146a, are located predominantly in the nucleus. The spliced transcripts are present in the nucleus and the cytoplasm. Since the first processing step of pri-miRNAs takes place in the nucleus [32,33], both nuclear unspliced and spliced transcripts can serve as pri-miRNA templates. This was supported by presence of both unspliced and spliced BIC transcripts in the DGCR8-containing Microprocessor complex. Overexpression constructs containing either the exonic 5’ flanking sequence or the intronic 5’ flanking sequence of pre-miR-155 both resulted in a marked miR-155 induction. Consistent with these findings we also observed a marked induction of miR-155 in cells with overexpressed full-length spliced BIC transcripts. These data also indicate that the Microprocessor complex can use unspliced and spliced BIC transcripts for processing to pre-miR-155. Since the unspliced BIC transcripts are much more abundant and are almost exclusively located in the nucleus, we conclude that unspliced nuclear BIC transcripts are the primary template for miR-155 processing in B-cell lymphoma. In contrast to pri-miR-155, the spliced transcripts are the most abundant form of pri-miR-146a and, for part of the cell lines of pri-miR-22. Unspliced pri-miR-146a showed very low levels in all cell lines. This might indicate that the unspliced pri-miR-146a is directly used for processing to pre-miR-146a or for splicing. The level of spliced pri-miR-146a might thus represent the level of pri-miRNA that was not used for miRNA processing. Alternatively, despite the partial cytoplasmic location, the spliced transcripts can still be the most important source for the biogenesis of mature miR-146a. Overexpression of miRNAs from constructs that do not contain introns show very effective processing to mature miRNAs, similar to our results using the BIC constructs. This indicates that although splicing enhances processing [12,15], it is not required to allow efficient processing to mature miRNAs. Pawlicki et al. showed that overexpressed pri-miRNAs that are artificially prematurely released from the transcription site accumulate in the nucleoplasm and are not efficiently processed to pre-miRNA [13,34]. These studies implicate that spliced pri-miRNA transcripts may be less efficient templates for the miRNA processing machinery when released from the transcription site. Effective in vitro processing of pri-miRNAs using whole-cell extract or immunoprecipitated Microprocessor [6] indicates that presence of the transcript at the transcriptional start site is not required for pre-miRNA release. Although we do not know if spliced exonic pri-miRNA transcripts are released from the transcription start site before miRNA processing, our data clearly show that spliced transcripts can be used for miRNA processing.

We observed that induction of BIC using three different constructs was variable. However, the induction of mature
miR-155 was strikingly similar in the three cell lines. Thus, induction of higher BIC transcripts levels did not result in higher miR-155 levels. This suggests that the miR-155 levels are regulated in these cell lines characterized by very low endogenous miR-155 levels. Notably, the level of miR-155 obtained with these three constructs was still ~10 fold lower than the highest observed endogenous miR-155 levels in OCI-Ly3 cell line. An alternative explanation could be that factors required for miRNA biogenesis become limiting and preclude induction of higher levels.

For BIC, we demonstrated that part of the spliced transcripts are exported to the cytoplasm and are thus not available for processing. Similarly, spliced pri-miR-22 and to a lesser degree spliced pri-miR-146a are exported to the cytoplasm. Alteration of the efficiency of splicing and nuclear export of spliced pri-miRNA transcripts may rapidly change the amount of pri-miRNA available for miRNA processing and therefore serve as a mechanism to regulate mature miRNA levels. Consistent with this hypothesis, we observed differences in the ratio of unspliced to spliced BIC transcript levels upon PMA/Ionomycin treatment. Cellular conditions and external stimuli may thus affect exonic miRNA levels by inducing changes to the amount of unspliced pri-miRNA used for miRNA processing at the expense of the amount of unspliced transcript available for splicing.

Both unspliced and spliced exonic miRNA transcripts can be used as substrate for miRNA processing. The level and ratio of spliced and unspliced transcripts, their cellular location and the processing efficiency together determine which form is the most likely endogenous pri-miRNA. For exonic miRNA processing studies it is important to assess total transcript levels and not only examine either spliced or unspliced transcripts. Conflicting data as presented in the current literature concerning the processing efficiency of BIC may, at least partially, be explained by differences in the analyzed transcripts [25,26].

Many proteins were reported to inhibit or promote miRNA processing by binding to stem-loop of pri-miRNA and/or pre-miRNA (reviewed in 3). These proteins have been identified to regulate both intronic and exonic miRNA processing. KH-type splicing regulatory protein (KSRP) was shown to enhance miR-155 processing in mouse activated macrophages by binding to the terminal loop of both BIC transcript and pre-miR-155 [35]. Moreover, monocyte chemoattractant protein [MCP]-1-induced protein 1 (MCPIP1) was shown to suppress miRNA processing of a panel of miRNAs, including miR-155 and miR-146a, by induction of pre-miRNA terminal loops cleavage [36]. Some of these proteins, like KSRP or hnRNP A1, regulate both miRNA and mRNA processing [37-39]. These, and possibly other, regulatory proteins may thus regulate exonic miRNA levels by promoting either pre-miRNA cleavage or splicing of exonic pri-miRNA transcripts.

It is unclear whether cytoplasmic spliced pri-miRNA transcripts have a function in the cytoplasm. To date, the only known function of the three noncoding genes studied in this paper is being the host gene for the miRNAs. Splicing of the transcripts and the subsequent transport to the cytoplasm might serve as a mechanism to prevent processing to pre-miRNA (Figure 5). This is supported by the finding that upon inhibition of Dicer8 with shRNA in L1236 cells we saw a marked induction of the spliced BIC transcript, which resulted in a change of the unspliced/spliced BIC transcript ratio from 2.5 to 0.3 (data not shown). This indicates that when pri-miRNA processing is inhibited, splicing of unspliced BIC transcript is enhanced. Another possible role of the cytoplasmic spliced transcripts is that they may function as competing endogenous RNA (ceRNA) transcripts for the mature miRNAs. Mir-155 and mir-155* sequences are highly complementary and the BIC transcript is a predicted mir-155 target by the miRanda-mirSVR and PITA algorithms (http://www.microrna.org [40]; http://genie.weizmann.ac.il/pubs/mir07/mir07_prediction.html [41]). Up to date, various transcripts were shown to function as ceRNA, e.g. protein-coding transcripts, pseudogenes, and long noncoding RNAs [42-44]. Spliced pri-miRNA transcripts of exonic miRNAs could prevent binding of the mature miRNA to their endogenous protein-coding target genes and thereby prevent efficient knock down of the target proteins. This would be a novel mechanism, by which cytoplasmic pri-miRNA transcripts function in a negative feedback-back loop to regulate miRNA function. Another possibility is that pri-miRNAs are processed in the cytoplasm, similar to Drosha-mediated processing of viral pri-miRNAs that was shown to take place in the cytoplasm [45]. In these cells relocation of Drosha to the cytoplasm and the subsequent pri-miRNA processing was triggered by viral infection. This implicates that Drosha relocation might potentially also occur in normal cells under certain conditions.

In conclusion, we showed that unspliced BIC, pri-miR-146a and pri-miR-22 transcripts were predominantly localized in the nucleus, although spliced transcripts are more abundant in
some cases. We also showed that spliced BIC, pri-miR-146a and pri-miR-22 transcripts are partly localized in the cytoplasm and thus not fully available for processing to the mature miRNAs. Pre-miRNAs and spliced transcripts appear to be two mutually exclusive products of unspliced pri-miRNA transcripts of exonic miRNA. Splicing and transport to the cytoplasm may represent a novel mechanism to regulate cellular exonic miRNA levels and function.

Materials and Methods

Cell lines and treatment

Burkitt lymphoma cell lines (Ramos, DG-75, ST486, NAMALWA, Raji, Jiyoye) were purchased from ATCC (ST486) and DSMZ (other cell lines). Diffuse large B-cell lymphoma cell lines (OCI-Ly3, SU-DHL-4, SU-DHL-6, VER) were a kind gift of A. Rosenwald (University of Würzburg, Germany) (OCI-Ly3) [46,47], A. Epstein (UCLA, CA) (SU-DHL-4 and SU-DHL-6) [46,47] or were established in our laboratory (DEV) [54]. Primary BIC amplified from genomic DNA (s-intBIC) and P. Möller (University of Ulm, Germany) (U-HO1) [46,47], A. Epstein (UCLA, CA) (SU-DHL-4 and SU-DHL-6) [46,47] or were established in our laboratory (VER) [48]. Hodgkin lymphoma cell lines (L540, L591, L1236, DEV, KM-H2, HDLM-2, L428, U-HO1) were purchased from DSMZ (L540, KM-H2), were a kind gift of V. Diehl (University of Cologne, Germany) (L591 [49], L1236 [50], HDLM-2 [49], L428 [51,52]) and P. Möller (University of Ulm, Germany) (U-HO1) [53] or were established in our laboratory (DEV) [54]. Primary mediastinal B-cell lymphoma cell lines (KARPAS-1106P, MEDB-1) [55] were a kind gift of M. Dyer (University of Leicester, UK). Cell lines were cultured at 37°C under an atmosphere containing 5% CO2 in Iscove's Modified Dulbecco's Medium (OCI-Ly3) or RPMI-1640 (other cell lines) medium (Cambrex Biosciences, Walkersville, USA) supplemented with ultraglutamine (2 mM), penicillin (100 U/ml), streptomycin (0.1 mg/ml; Cambrex Biosciences), and 5% (L428), 20% (DEV, ST486, OCI-Ly3) or 10% (other cell lines) fetal calf serum (Cambrex Biosciences). DG-75, L428 and KM-H2 cells were treated for 24h with Phorbol 12-myristate 13-acetate (PMA)/Ionomycin (both Sigma-Aldrich, Saint Louis, MO) as previously described [25]. The PMA/Ionomycin treatment was performed in triplicate (DG-75 and L428) or quadruplicate (KM-H2).

BIC/pri-miR-155 constructs

The pcDNA3.1(+) plasmid containing full-length spliced BIC (fl-exBIC) was described previously [25]. The MXW-PGK-IRESGFP vector was a kind gift from C-Z. Chen (Stanford University, CA). The full-length spliced fl-exBIC insert was subcloned from the pcDNA3.1(+) vector to the MXW-PGK-IRESGFP vector using Pmel (pcDNA3.1(+) vector) and Hpal (MXW-PGK-IRESGFP vector) restriction enzymes. The miR-155 stem-loop and ~150nt flanking sequences were amplified from genomic DNA (s-intBIC) or cDNA (s-exBIC) using Taq polymerase. Primer sequences used for PCR were as follows, 5'-TGTCACCTCCACGTTTAAAAC-3' (forward, s-intBIC), 5'-AACCTACAGAACCTTAC-3' (forward, s-exBIC), 5'-GGCTTTATGCTTTCAATTC-3' (reverse, s-intBIC) and 5'-exBIC). An Xhol restriction site was added to the forward and an EcoRI site to the reverse primer to allow efficient cloning. PCR products were cloned to the retroviral MXW-PGK-IRES-GFP vector using standard laboratory procedures. The inserts were sequenced to confirm the correct sequences.

Retroviral transduction

To generate retroviral particles, Phoenix-Ampho packaging cells [56] were CaPO4 transfected with 37.5µg of MXW-PGK-IRES-GFP constructs (empty vector or vector containing one of the BIC constructs) in T75 flasks. Viral particles were harvested after two days and concentrated with Retro-X concentrator (Clontech, Saint-Germain-en-Laye, France) according to the manufacturer's protocol. Target cells were transduced with the virus by spinning at 2,000 rpm for 2hrs. Cells transduced with retroviral vectors were sorted for GFP using MoFlo sorter (Dako cytometry).

RNA isolation from total, nuclear and cytoplasmic fractions

Nuclear and cytoplasmic fractions were separated by adding 200µl of lysis buffer (140mM NaCl, 1.5mM MgCl2, 10mM Tris-HCl pH8.0, 1mM DTT, 0.5% Nonidet P-40) to pellets of ~4 million cells, followed by 5min incubation on ice and centrifugation for 3min at 4°C and 100g. The supernatant was harvested as the cytoplasmic fraction. The pellet containing the nuclei was washed twice with lysis buffer. 1ml of Qiazol (Qiagen, Carlsbad, USA) was added to the ~200µl of cytoplasmic fraction, to the nuclear pellet and to the total cell pellet.

Quantitative RT-PCR

Total RNA was isolated using Trizol (Invitrogen, Carlsbad, USA) according to the manufacturer's protocol for the cell lines. RNA samples were treated with DNase (Ambion, Foster City, CA). The RNA concentration was measured with a NanoDrop™ 1000 Spectrophotometer (Thermo Fisher Scientific Inc., Waltham, USA) and RNA integrity was evaluated by 1% agarose electrophoresis. cDNA was synthesized using 500ng input RNA, SuperScript II and random primers according to the manufacturers protocol (Invitrogen). The qPCR reaction contained SYBRgreen mix (Applied Biosystems, Foster City, USA), 300nM primers, and 1ng of cDNA in a total volume of 10µl. Levels of spliced, unspliced and total BIC as well as spliced and unspliced pri-miR-22, pri-miR-146a were normalized to HPRT. For cytoplasmic, nuclear and total fractions, qRT-PCR was performed as described above with slight modifications. We used the miRNeasy kit (Qiagen) including a DNase-treatment (Qiagen) for RNA isolation. RNA was diluted in the same way for total and cytoplasmic fractions and 5 times less for nuclear fraction to use similar amounts (~500ng) for cDNA synthesis. 1/500 of the cDNA was used for qRT-PCR. C, values of nuclear fractions were corrected for different RNA dilutions. Transcript levels in nuclear and cytoplasmic fractions were calculated relative to total fraction. Purity of nuclear fractions was confirmed with U3 and Xist and of the cytoplasmic fraction with rRNA-Lys. For qRT-PCR the following primer sequences used were: for unspliced BIC, 5'-AGCTTTATACCGCATGTGCATAC-3' (forward) and 5'-CAGATTTCCCATCCCTGGTTT-3' (reverse); for total BIC, 5'-AAATTCTTATGCGTCTACGTA-3' (forward) and 5'-
Localization of unspliced or spliced analysis. Levels of unspliced and spliced pri-miR-22, 5'-CTGCTCAGATCTTTCCCATTTTC-3' (forward) and 5'-CAGCCAGCGAGCTCCTAAAA-3' (reverse); for spliced pri-miR-146a, 5'-ATTTCACCGGCTTTCACCTTGATT-3' (forward) and 5'-GGCTTTTCAGAGATGGTGCAA-3' (reverse); for spliced pri-miR-146a, 5'-GACAGGAGACAGTACGACAAAG-3' (forward) and 5'-CAGCCAGCGAGCTCCTAAAA-3' (reverse); for Xist, 5'-GTCTTTTCTTTGACCCAGAA-3' (forward) and 5'-GAGCCCTGGCATTTTTTTCC-3' (reverse); primers for spliced BIC, HPRT, tRNA-Lys and U3 were described previously [21,57,58]. Localization of unspliced or spliced transcript-specific qRT-PCR products are indicated in Figure 2A. qRT-PCR for miR-155, miR-22, miR-146a, miR-16, miR-191, tRNA-Lys and RNU48 was performed using miRNA qRT-PCR assays (Applied Biosystems, Foster City, USA) as described previously [59]. Reverse transcription (RT) primers specific for a miRNA and RNU48 (control) were multiplexed in 15µl RT reactions containing 1µl of each RT primer. The miRNA levels were normalized to the RNU48 levels. Mean cycle threshold (C\text{t}) values for all genes were quantified with the SDS software (version 2.1). Relative expression levels were calculated as 2^{-\Delta\Delta C_{\text{t}}}.

**DGC8 immunoprecipitation**

Immunoprecipitation of DGC8-containing Microprocessor complexes was performed as described previously for Ago2-IP with some slight modifications [60]. Briefly, ∼30 million cells were lysed with 100 µl of the same lysis buffer as used for the cellular fractionation and sonicated for 5 seconds. lysate was incubated with protein A Sepharose beads (GE Healthcare) coated with anti-DGC8 antibody (ab90579, Abcam, Cambridge, UK) at 4°C overnight. Mouse IgG antibody was used as a negative control (Millipore BV, Amsterdam, The Netherlands). After washing the beads, RNA was harvested for qRT-PCR analysis and protein lysates were prepared for Western blot. Western blot for DGC8 was performed as described previously [60], using a 1:2000 dilution of the DGC8 antibody at 4°C overnight. Total RNA was isolated with miRNAeasy Kit (Qiagen) according to manufacturer’s protocol. RNA from DGC8-IP and IgG-IP fractions of Jiyoye, U-HO1 fl-exBIC and Ramos fl-exBIC cells was used for qRT-PCR analysis. Levels of unspliced and spliced BIC transcripts in DGC8-IP and IgG-IP fraction were calculated relative to tRNA-Lys and fold increase in the DGC8-IP fraction was calculated for each sample.

**Supporting Information**

**Figure S1.** The endogenous levels of mature miRNAs, unspliced and spliced pri-miRNAs of miR-155, miR-22 and miR-146a in B-cell lymphoma. The levels of miR-155 (A), miR-22 (C) and miR-146a (E) in the cell line panel were sorted from low to high. Unspliced and spliced transcripts levels of BIC (B), pri-miR-22 (D) and pri-miR-146a (F) in 20 B-cell lymphoma cell lines. In 17 out of 20 cell lines the levels of unspliced BIC transcripts were higher than the levels of spliced BIC transcripts. Spliced pri-miR-22 transcript levels were higher than unspliced pri-miR-22 transcript levels in 9, similar in 9 and lower in 2 of the analyzed cell lines. In all tested cell lines, levels of spliced pri-miR-146a were much higher than unspliced pri-miR-146a transcripts. Levels of BIC, pri-miR-22 and pri-miR-146a were normalized to HPRT and levels of miR-155, miR-22 and miR-146a were normalized to RNU48. (TIF)

**Figure S2.** Validation of nuclear and cytoplasmic fraction purity. (A) Cytoplasmic control, tRNA-Lys, was mostly present in the cytoplasmic fraction of L428, L540 and Jiyoye cells. From the two nuclear controls, U3 and Xist, U3 (B) showed slightly higher level in the nucleus and Xist (C) was exclusively nuclear in the two female cell lines, L428 and L540. Similarity, for Ramos EV and Ramos fl-exBIC, tRNA-Lys (D) was more abundant in the cytoplasm and U3 (E) more abundant in the nucleus. Amount of transcripts was calculated relative to the total fraction and corrected for the amount of RNA per cell. Average of 3 experiments was shown. (TIF)

**Figure S3.** Validation of DGC8-IP fractions. (A) Western blot for DGC8 in Ramos fl-exBIC cells. DGC8 was pulled down with anti-DGC8 antibody and not with the non-specific IgG control. (B) Levels of unspliced and spliced BIC transcripts in total fractions of Jiyoye, Ramos fl-exBIC and U-HO1 fl-exBIC cells. Levels of exogenous spliced BIC transcripts were much higher than endogenous unspliced BIC transcripts for Ramos and U-HO1. (C) Relative amounts of unspliced and spliced BIC transcripts in DGC8-IP fractions were similar in Jiyoye cells. In the Ramos and U-HO1 cell lines, exogenous spliced BIC transcripts were much more abundant in the DGC8-IP fraction than unspliced BIC transcripts. (TIF)

**Figure S4.** Induction of miR-22 and miR-146a upon cellular activation. Fold induction in levels of miR-22 (A), unspliced and spliced pri-miR-22 (B) in cells treated with PMA/ionomycin (P/I). Levels of miR-22 were increased in 2 out of 3 cell lines. Levels of unspliced and spliced pri-miR-22 were increased in all tested cell lines. (C) No difference in pri-miR-22 unspliced/spliced ratio was observed upon P/I treatment. Mature miR-146a (D), unspliced and spliced pri-miR-146a (E) transcript levels were also higher P/I-treated cells. (F) Pri-miR-146a unspliced/spliced ratio was not altered upon cellular activation. No differences in levels of intronic miRNAs, miR-16 (G) and miR-191 (H), or intergenic let-7a (I) were observed. Average of 3 experiments was presented. Student’s t-test was used to determine p values (*p<0.05, **p<0.01, ***p<0.001, ns - not significant). (TIF)

**Author Contributions**

Conceived and designed the experiments: IS-P JK B-JK SP AB. Performed the experiments: IS-P DJ NH GK. Analyzed the
References


