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Characterisation and discovery of novel miRNAs and moRNAs in JAK2V617F mutated SET2 cells / Bortoluzzi, S., Bisognin, A., Biasolo, M., Guglielmelli, P., Biamonte, F., Norfo, R., Manfredini, R., Vannucchi, A.M.. - In: BLOOD. - ISSN 0006-4971. - ELETTRONICO. - 119:13(2012), pp. e120-e130. [10.1182/blood-2011-07-368001]

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28/06/2026 19:35

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blood

Prepublished online January 5, 2012;
doi:10.1182/blood-2011-07-368001

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Blood (print ISSN 0006-4971, online ISSN 1528-0020), is published weekly by the American Society of Hematology, 2021 L St, NW, Suite 900, Washington DC 20036.
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Characterization and discovery of novel miRNAs and moRNAs in *JAK2V617F* mutated SET2 cells

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Right running head: miRNA discovery in *JAK2V617F* mutated SET2 cells

Left running head: BORTOLUZZI ET AL

Scientific category: Myeloid neoplasia

ABSTRACT

In order to gain insights into a possible role of microRNAs in myeloproliferative neoplasms, we performed short RNAs massive sequencing and extensive bioinformatic analysis in the *JAK2V617F* mutated SET2 cell line. Overall, 652 known mature miRNAs were detected, of which 21 were highly expressed, thus being responsible of most of miRNA-mediated gene repression. microRNA putative targets were enriched in specific signaling pathways, providing information about cell activities under massive post-transcriptional regulation. The majority of miRNAs were mixtures of sequence variants, called isomiRs, mainly due to alternative, non-canonical processing of hairpin precursors. We also identified 78 novel miRNAs (miRNA*) derived from known hairpin precursors. Both major and minor (*) form of miRNAs were expressed concurrently from half of expressed hairpins, highlighting the relevance of miRNA* and the complexity of strand selection bias regulation. Finally, we discovered that SET2 cells express a number of miRNA-offset RNAs (moRNAs), short RNAs derived from genomic regions flanking mature miRNAs. We provide novel data about the possible origin of moRNAs, while their functional role remains to be elucidated. Overall, this study shed light on the complexity of microRNA-mediated gene regulation in SET2 cells and represents the basis for future studies in *JAK2V617F* mutated cellular models.

INTRODUCTION

The *JAK2V617F* mutation, that occurs in most patients with polycythemia vera and $\cong 60\%$ of essential thrombocythemia and primary myelofibrosis¹, is considered integral to the pathogenesis of myeloproliferative neoplasms (MPN), although additional, antecedent mutations are required for a MPN to develop²⁻⁴. The disease can be reproduced in mice expressing the *JAK2V617F* allele⁵. Cells harboring the *JAK2V617F* mutation display autonomous activation of several cell signaling pathways, particularly JAK/STAT, and proliferate and mature in a cytokine-independent manner⁶. Recent information highlighted that deranged epigenetic gene regulation^{7,8} and abnormal expression of microRNAs⁹⁻¹¹ also contribute to the pathogenesis of MPN.

microRNAs constitute a large class of non-coding RNAs that act as post-transcriptional regulators of gene expression. Since dysregulation of miRNA expression plays a critical role in genetic and multifactorial disorders and most, if not all, human cancers¹², miRNAs are considered prominent tumor markers, oncogenes (oncomiRNAs), relevant targets for therapy or therapeutic agents depending on the specific context¹³. We and others have identified abnormally expressed microRNAs in CD34⁺ and blood cells of MPN patients, some of which have a JAK2-dependent pattern of expression⁹⁻¹¹. One of these, miR-16 is over-expressed in polycythemia vera CD34⁺ cells and contributes to the expansion of erythropoiesis¹⁴.

There is an intrinsic complexity of microRNA system that derives both from the large number of coding RNAs that represent possible targets of a single mature microRNA and the variety of mature different miRNA molecules that originate from a single genetic locus. Both for intragenic and intergenic (single or clustered) miRNAs, primary miRNAs are transcribed, edited and cleaved in the nucleus to generate hairpin precursors that are exported to the cytoplasm where maturation occurs¹⁵. Two different mature miRNA sequences can derive from the same precursor hairpin: a major form (e.g. hsa-let-7c), that is the most stable

and prevalent, and a minor form (e.g. hsa-let-7c*). The pre-miRNA hairpin is cleaved in the cytoplasm by Dicer to produce miRNA duplex (~22 nt)¹⁶ that is incorporated in the RISC complex. The RISC recognizes the duplex, unwinds it, selects the guide miRNA strand (while degrading the passenger strand) and mediates recognition and silencing of target coding RNAs. Both miRNA forms can be expressed and are functionally effective, while sometimes the “minor” form prevails¹⁷⁻¹⁹. Major and minor forms are associated to different sets of target coding mRNAs, contributing uniquely to the regulation of cell activities.

Furthermore, deep sequencing technologies (RNAseq) led to discover a great number of novel miRNAs in the last years²⁰, including in normal and malignant blood cells^{21,22}. It has also been shown in animals (from *D. melanogaster* to humans) and plants that mature miRNA sequences are not invariant; rather, they constitute a mixture of miRNAs variants, i.e. isoforms heterogeneous in 5' and 3' ends or with non-template 3' additions of nucleotides, that are collectively called isomiRs^{23,24}. These variants, all potentially active, target only partially overlapping sets of coding mRNAs. Finally, a novel class of miRNA-related RNAs, micro-RNA offset RNAs (moRNAs), has been recently identified by RNAseq (see²⁵ for a brief but detailed review).

moRNAs were first reported in the simple chordate ascidian *Ciona intestinalis*²⁶, as ~20nt long RNAs that originate predominantly from the 5' arm of the pre-miRNA, regardless of the position of the major miRNA, suggesting that moRNA and miRNA biogenesis may be linked but not necessarily interdependent. The expression of moRNAs in *C. intestinalis* is developmental regulated and their abundance sometimes exceeds the corresponding mature miRNA. Initially *C. intestinalis* moRNAs were considered by-products of a miRNA processing machinery with intrinsic properties²⁶. However, shortly thereafter, Langerberger et al. reported moRNAs, derived from 78 miRNA loci, as being weakly expressed in human prefrontal cortex²⁷, while Taft et al.²⁸ showed that moRNAs were enriched in the nucleus in the human leukemia cell line THP-1 and almost exclusively derived from the 5'-arm, regardless the position of the prevalent miRNA. Some moRNAs were even more expressed than the prevalent miRNA of the same locus. Thereafter, moRNAs (e.g. hsa-miR-410 5'moR,

has-miR-326 5'moR) were discovered in solid tumors²⁹.

In this study, we employed massive sequencing of short RNAs with the aim to discover novel miRNAs in known hairpin precursors, characterize and quantify isomiRNAs, and identify moRNAs in SET2 cells, a *JAK2V617F* mutated cell line. Furthermore, the expression relationships between sister miRNA pairs (miR/miR* of the same hairpin), between sister moRNAs and between moRNAs and the corresponding miRNAs were investigated. The effects of a JAK2 inhibitor on the modulation of miRNA expression in SET2 cells were also evaluated. This report shows an unforeseen complexity of small RNAs in this cellular system and represents the starting point for further functional studies and systematic analysis in primary MPN cells.

MATERIALS AND METHODS

RNA isolation, short RNA library construction and sequencing

Total RNA, prepared from exponentially growing SET2 cells with miRNeasy reagents (Qiagen), was used to generate a small RNA library according to standard Illumina procedure (Supplemental Methods). The purified cDNA library was used for cluster generation on Illumina's Cluster Station and sequenced on Illumina GAIIx. Raw sequencing reads were obtained using Illumina's Pipeline v1.5 software following analysis of sequencing image by Pipeline Firecrest Module and base-calling by Pipeline Bustard Module. Library construction and sequencing were performed at LC Sciences (Houston, Tx; www.lcsciences.com)

Short RNAs sequencing data processing

A detailed description of the computational pipeline employed for data handling and analysis is reported in the Supplemental Methods, including a flow-chart outline of study procedures (Figure S1).

KEGG pathways enrichment

KEGG pathway enrichment for known miRNAs was obtained using DIANA-miRPath webtool³⁰ based on TargetScan5 miRNA target predictions. mirPath is used to identify molecular pathways potentially altered by the expression of single or multiple microRNAs through enrichment analysis of multiple microRNA targets and comparison of each set of microRNA targets to all KEGG pathways. The combinatorial effect of co-expressed microRNAs is taken into account by simultaneous analysis of multiple microRNAs. Only pathways including at least five genes and displaying an enrichment P-value of >0.001 were considered significant.

New miRNA target prediction

Genes target of new miRNAs were predicted by TargetScan using as input new miRNA sequences and the set of 3'UTR sequences of all human transcripts and orthologs in 23 species (TargetScan database v5.2) (for details, see Supplemental Methods).

Analysis of miRNA expression in SET2 cells exposed to the JAK1/JAK2 inhibitor INC424.

SET2 cells were incubated with increasing concentration of INC424/Ruxolitinib for 3-6 h and RNA was purified. Affymetrix Genechip miRNA array 2.0 was used to identify miRNA species showing a differential expression in treated cells compared with control cells (for a detailed description of procedure, see Supplemental Methods).

RESULTS

The Illumina GAIIx sequencing of the small RNA library from SET-2 cells produced 32,760,003 reads which, after extensive preprocessing and quality control, were reduced to 27,906,609 reads, 85% of sequenced reads. A first mapping phase aimed at discarding

contaminations and repeats, yet tolerating for possibly unknown miRNA loci, produced 22,167,999 reads (68% of raw data). These were mapped to “extended hairpins” in order to identify and quantify known miRNAs and for discovery and characterization of novel isomiRs and other miRNA-associated expressed RNAs. In total, 1,421 known hairpin precursors, corresponding to 1,731 known mature miRNA sequences and including a number of miRNAs recently discovered by RNAseq, were finally considered (Table S1). A detailed description of sequencing data processing is provided in Supplemental Methods. Raw sequencing data and processed files are available at GEO database (GSE30672).

Known miRNAs expressed in SET2 cells

A total of 652 known miRNAs were found expressed in SET2 cells, with expression levels ranging from 10 to 2,268,333 (mean 29,830, median 613); 300 and 124 miRNAs presented read counts of at least 10^3 and 10^4 , respectively (Suppl. Table 2). Twenty-one only highly expressed miRNAs accounted for 70% of known miRNA expression (Table 1) and are thus predicted to account for most of miRNA-mediated gene repression in SET2 cells. Table S3 reports KEGG pathways significantly enriched in genes predicted target of 21 most expressed miRNAs. These include pathways anticipated to have functional relevance for MPN-associated cellular abnormalities, such as the MAPK signaling pathway, TGF-beta signaling pathway, mTOR signaling pathway, Wnt signaling pathway. These findings produced meaningful insights about cell activities that are under the control of massively expressed miRNAs in SET2 cells. Table S4 reports functional insights for the 21 most expressed miRNAs.

miRNA sequence heterogeneity (isomiRs)

The majority of expressed mature miRNAs were not represented by a unique sequence corresponding to that annotated in miRBase. We evaluated the whole group of reads belonging to each miRNA, including the “classic” mature sequence annotated in miRBase (“exact” alignment) as well as those reads perfectly matching the precursor but overlapping the mature position by 3nt (longer/shorter), those presenting 1 mismatch (1-

Mismatch), and those presenting two mismatches at the 3' end (2-3'-Mismatches) (see Supplemental methods for details).

Considering the whole set of variants presenting at least 10 reads each, 636 miRNAs were identified: 232 (36%) appeared "invariant" whereas the remaining 404 (64%) represented a mixture of 2 to 6 sequence variants (Figure S2). To be conservative, we considered as "biologically meaningful" sequence variants (isomiRs) accounting for at least 10% of the total per miRNA read count, calculated over all variants (Figure S2). In this way, out of 644 miRNAs represented, 224 miRNAs (35%) are "invariant" and 420 (65%) are associated to 2 to 6 isomiRs each. Nevertheless, it is interesting that some of miRNAs considered "invariant" are associated to an unique major sequence as well as to other sequences that have not been annotated since none of them satisfied the required number of reads.

We reasoned that the presence of isomiRs could be particularly relevant for miRNAs expressed at the highest level. In Figure 1A we report the distribution of number of isomiRs per miRNA separately for the 25% most Expressed miRNAs (MEms) and for the remaining weakly expressed miRNAs. MEms were associated to one to four isomiRs. The contribution of the major isomiR (i.e., the isomiR with the highest read count among all isomiRs of the miRNA) to total count was highly variable, accounting for a fraction of total count which was inversely related to the total number of isomiRs per miRNA (Figure 1B). Specifically, the contribution of the major isomiR averaged 52% in case of miRNAs with more than one isomiRs and 83% in "invariant" miRNAs. As known, 5' and 3' regions of mature miRNA sequence play a different role in target recognition: in case of canonical target sites, the seed region, whose pairing to the target is crucial, is included in the 5' of mature miRNA. It is conceivable that isomiRs with different seed sequence recognize and regulate different targets. Thus, for each MEm, we considered the 5' and 3' half sequences separately, and classified expressed isomiRs according to the observed difference between the isomiR sequence and the "classic" mature miRNA (mismatch or length difference) in the involved region. Out of 819 isomiRs, 648 (79%) differed in the 3' region, 64 in the 5' region (8%) and 107 in both (13%). No "Mismatch" isomiRs were observed regarding the 5' region. Table S5 includes

details about isomiR sequences and counts for the group of highly expressed miRNAs. Figure 2 reports the relative contribution of isomiRs for the 21 most expressed miRNAs in SET2 cells.

In the MEM set, 323 isomiRs were identified: 140 (43%) were coincident with the miRNA sequence reported in miRBase (classic isomiR), 154 (48%) represented sequence variants with longer or shorter 5' and 3' ends, and 29 (9%) showed one nt difference versus the classic isomiR. Interestingly, we found that the major isomiR was not coincident with the classic sequence annotated in miRBase in 53 of 161 highly expressed miRNAs (33%). Besides, the isomiR corresponding to the sequence annotated in miRBase was not detected in 21 (13%) of the miRNAs expressed in SET2 cells.

We then considered 113 highly expressed miRNAs associated to more than one isomiR to understand how much mismatch alignments contributed to sequence variability. We found that 87 of these (77%) did not include isomiRs with single nt substitutions respective to the sequence annotated in miRBase, whereas only a minority, which may arise from SNPs, RNA editing as well as from sequencing errors, included one or two isomiRs aligning with one mismatch to the hairpin precursor (24 and 2 miRNAs respectively). In terms of total expression, isomiRs with mismatch alignments contributed only to < 3% of read count. Classic isomiRs and isomiRs showing 5' and 3' length variability almost equally contribute to 97% of total read counts of highly expressed miRNAs. Therefore, we concluded that the most important sequence variation is represented by 5' and 3' length variability, possibly occurring as a consequence of alternative, non- canonical, regulated processing of the precursor sequence.

Novel miRNAs expressed in SET2 cells were discovered in known hairpin precursors

For novel miRNA discovery, we operationally defined as “expressed RNA elements” (ERE) those discrete hairpin regions covered by overlapping reads with a minimum count of 10 and a start position within 4 nt each from the following one. We found that a discrete number of regions located outside known mature miRNAs were expressed from detectable

to high level; a number of EREs able to pair with known miRNAs in the most probable duplex produced by Dicer processing of the hairpin structure were identified. Specifically, we considered 943 hairpins associated to only one annotated mature miRNA, whereas an additional 478 included two known sister mature miRNAs.

The analysis of known hairpin precursors associated to only one known miRNA produced a set of 78 novel miRNAs expressed in SET2 cells (Table S6). Plots in Figure 3 show, for the four most expressed new miRNAs (hsa-miR-1307*, hsa-miR-376a-2*, hsa-miR-382* and hsa-miR-539*), the number of reads aligned per nucleotide position. This information was integrated with hairpin sequence and folding data to define new miRNA sequences. Table 2 reports name, sequence and expression level of novel miRNAs presenting a read count of >500. Expression levels of new miRNAs ranged from 10 to 72,670 (mean 1471, median 63) and were significantly lower than known miRNAs (Two Sample t-test of mean equality p-value =6.521e-06) (Figure S3). Nevertheless, 11 new miRNAs (14%) showed an expression level higher than the median value observed for known miRNAs. In particular, hsa-miR-1307*, hsa-miR-376a-2* and hsa-miR-382* resulted very highly expressed, at level even >75% of known miRNAs.

TargetScan custom target predictions (See Methods) for 15 novel miRNAs with a read count >500 are included in Table S7. Specifically, conserved and not-conserved target sites were predicted using TargetScan and filtered according to the context score. Only conserved target sites associated to top 25% scores and non-conserved sites included in top 5% scores were reported.

Sister miRNA expression prevalence

Considering known and new miRNAs expressed in SET2 cells as a whole, we found that both miRNA and miRNA* were expressed concurrently in 260 hairpins, corresponding to about one half of those with at least one miRNA expressed (Table 3). miRNA and miRNA* of the same hairpin, called a sister miRNA pair, have different sequences, thus targeting different

sets of coding RNAs and contributing uniquely, but possibly in a coordinate way, to transcriptional regulation. When increasing thresholds of read count were applied in order to consider a miRNA as being “expressed”, the fraction of hairpins producing concurrently miRNA and miRNA* decreased (Table 3), while the fraction of hairpins producing a meaningful quantity of both miRNAs remained considerable at all thresholds.

We observed no strand prevalence in expressed miRNAs (Figure S4). Out of 277 hairpins expressing only one miRNA, 47% and 53% expressed only the 5’ or 3’ miRNA (130 and 147), respectively. Regarding 260 hairpins expressing both sister miRNAs, the most expressed miRNA was the 5’ or 3’ form in 135 and 125, respectively. A considerable fraction of concurrently expressed miRNA pairs showed comparable levels: 66 (25%) and 38 (14%) of concurrently expressed pairs were associated to an absolute $\log_2(\text{ratio})$ of expression values not higher than 2 and 1, respectively.

miRNA-offset RNAs (moRNAs) identification in known hairpin precursors

We discovered ERE also outside known and novel miRNAs. These were classified, as explained in Supplemental Methods, as 5’ moRNAs, 3’ moRNAs and expressed loops (Figure 4). In particular, we identified 58 moRNAs expressed from 56 hairpins, at moderate to high level (mean 127, median 52; Tables S7, S8). The expression level of 16 and 4 moRNAs exceeded 100 and 500, respectively (Table 4). Three hairpins were associated each to two moRNAs expressed (Table S9, Figure 4); of these, hsa-mir-106b is an example of a “five-phased” precursor, with detected expression for 5 different regions. Figure 5 shows, for extended hairpins of the four most expressed new moRNAs, the plots of number of reads aligned against nucleotide position of extended hairpin sequences and predicted moRNA sequences.

We observed a considerable prevalence of 5’ moRNAs: 95% of moRNAs derived from the 5’ arm of the hairpin precursor (n=55 versus 5 for the 3’ moRNAs). The average expression level of 5’ moRNAs (129) was higher than 3’ moRNAs (89), although it did not reach the

significance level. Of interest, the prevalence of 5' moRNAs was not dependent on the type of the most expressed miRNA (Figure S5A).

Expressed miRNA length ranged from 16 to 27 nt, with an average of 21, at variance with moRNA sequences that were less variable in length, ranging from 18 to 25 nt, average of 20.2.

In order to derive insights concerning moRNA biogenesis, we considered the position of moRNA sequences relatively to both the miRNA hairpin precursor (Figure 6A) and the mature miRNA from the same hairpin arm (Figure 6B). Regarding the region of the hairpins from which moRNAs derived we noticed that 8 of 55 5' moRNAs were included in the "classic" hairpin precursor sequence while only one belonged to the region of 30 nt flanking the hairpin, whereas 46 (84%) were partially overlapping the hairpin 5' border. This indicates that moRNA sequence spanned the canonical Drosha cutting site and supports a role for non-canonical Drosha cleavage in moRNAs biogenesis. Moreover, we considered the positions of 50 couples of concurrently expressed 5' moRNAs and 5' miRNAs in extended hairpins. It is worth notice that 5' moRNAs and 5' miRNAs were adjacent in 27 pairs (54%): the miRNA sequence started exactly after the moRNA end. In this case, the two elements might derive from a single cutting step of a common precursor sequence. Conversely, in only one pair (2%) the two sequences were disjointed and the miRNA sequence started at least one nt after the moRNA end. Finally, 22 moRNAs (44%) overlapped with the nearby miRNA sequence for 1 to 8 nucleotides, indicating the presence of pairs of moRNAs and miRNAs that were likely produced alternatively from the processing of the same precursor.

miRNA and moRNA evolutionary conservation

Mean evolutionary sequence conservation in placental mammals of both detected miRNAs and moRNAs in SET2 cells varied widely from 0.0 to 1.0, representing extreme values of the conservation measure. Both distributions of conservation values are considerably negatively skewed. miRNA observed mean and median conservation values were 0.68 and 0.99,

respectively. Figure S6 shows the relationship between miRNA expression and average sequence conservation in placental mammals. In spite of high conservation variation, highly expressed miRNAs do not include poorly conserved elements. Conservation levels of 5' and 3' miRNA groups were almost the same (p-value= 0.83). Besides, the paired comparison of 5' and 3' miRNAs of the same hairpin highlighted a slight but significant (p-value 0.001) higher conservation of 3' miRNAs. Mean and median mRNA conservation scores were 0.80 and 0.99, respectively, no significantly different from miRNAs (p=0.165).

Changes in miRNA expression in SET2 cells treated with Ruxolitinib

Information concerning expression of different miRNA species in SET2 cells might represent a starting point for studies aimed at identifying set of miRNAs that lie in the JAK2 regulatory pathway. To this end, SET2 cells were treated with the JAK2 inhibitor INC424/Ruxolitinib and subjected to miRNA quantification using microarrays. INC424/Ruxolitinib caused a dramatic reduction of phosphoSTAT5 and Pim-1 mRNA, known targets of activated JAK2 (Figure 7) supporting the effectiveness of inhibition. By using stringent selection criteria (see Supplemental Methods) we identified 9 microRNAs among those identified by RNASeq in untreated cells that showed significant dose-dependent modulated expression (hsa-let-7c, hsa-miR-1299, hsa-miR323-3p were up-regulated, while hsa-miR-330-5p, hsa-miR-548, hsa-miR-665, hsa-miR-935u, hsa-miR-381, hsa-miR-92a-1* were down-regulated) (Figure 7). miRNA expression data are available at GEO (GSE33809).

DISCUSSION

In this study, we characterized the fraction of short RNAs expressed in SET2 cells, a *JAK2V617F* mutated cell line established from a patient with leukemic transformation of essential thrombocythemia, by exploiting deep sequencing data analyzed in integration with different levels of genomic information and metadata concerning known hairpin loci. We focused on known miRNA loci by considering 1,421 known hairpin precursors and 1,731 known mature miRNAs, including hundreds of new miRNAs discovered by massive sequencing

approaches. Sequencing data were processed and filtered with very stringent criteria, based on sequence length and quality. Reads mapping was obtained by using a quality-based method first and with a new method based on comparative mapping of reads to the human genome and to hairpin precursors later in order to filter out contaminant RNAs from repetitive loci.

One accomplishment of this study is the production of the first catalog of known miRNAs in SET2 cells and the identification of most expressed ones, which are conceivably responsible of most miRNA-mediated gene repression. Considering predicted targets of highly expressed miRNAs, different signaling (and biochemical) pathways were identified, obtaining interesting clues about possible regulatory circuits that are deregulated in SET2 cells (Table S4). For instance, we noticed that several among the top 25% expressed miRNAs belonged to two clusters, the miR-106b-25 (located on chr 7q22.1, that includes miR106b, miR-93 and miR-25) and the miR-17-92 (located on chr 13q31.3, that includes miR-17, miR-19b, miR-20a, and miR-92a, miR19a, miR18b); six of these miRNAs were among the 21 mostly expressed miRNAs. Recent evidence support the contention that uncontrolled expression of both or either these two clusters results in the loss of physiologic control of cell cycle arrest and apoptosis by transforming growth factor- β contributing to oncogenesis³¹.

The expression of known mature miRNAs from sequencing data has been estimated with a method able to correct for multiple mapping issues. Reads multiple mapping might arise from several reasons largely attributable to miRNA loci redundancy and similarity, to the existence of miRNA families, as well to reads-to-reference sequence mapping choices. The problem of mapping quality was considerably overlooked by different studies that recently exploited RNA-seq for miRNAs and isomiRs discovery. It has been shown³² that short reads are prone to map to multiple genomic loci with an equal number of mismatches (or even without mismatches), in particular among multicopy miRNA precursors (identical mature miRNAs may be produced by different hairpins, transcribed from different loci) and miRNA

families. Other studies considered only reads with unique mapping³³, although a too restrictive mapping settings may affect quantitative estimation of multicopy miRNAs. Basically, we implemented a “multiple-mapping corrected read count” to quantify correctly expression levels relative to both hairpin and mature miRNAs, also including multilocus miRNAs and miRNA families.

Another main finding of the study regards mature miRNA sequence variation. We showed that mature miRNAs were not represented by a unique sequence, i.e. the one annotated in miRBase, rather they were found as mixtures of sequence variants, called isomiRs, that derive mainly from non-canonical processing of hairpin precursors. Only one third of expressed miRNAs were associated to a unique isomiR corresponding to known mature miRNA sequence, with two third being associated to different sequences. Surprisingly, in 33% of the miRNAs with at least two isomiRs, the most expressed isomiR was not identical to the miRBase-annotated mature miRNA sequence. Using a conservative approach by focusing only on highly expressed miRNAs and considering merely those isomiRs accounting for at least 10% of total miRNA expression, we showed that 60% of miRNAs are associated to 2-4 isomiRs, that differ from the most expressed isomiR only for 5' and 3' sequence length but align exactly to the hairpin precursor. This might imply that most isomiRs arise as a consequence of alternative processing of miRNA precursors. Interestingly, the sequence variability of isomiRs regards prevalently the 3' region of mature miRNAs; however, 8% of isomiRs differ from the “classic” miRNA sequence in the 5' region, possibly impacting on target recognition and/or regulatory activity and strength. Future evaluations of isomiR quality and proportion in different cell types will help to clarify if and how much this miRNA biogenesis feature is cell- and context-specifically regulated.

In the second part of the study, we exploited the discovery power of RNA-seq to identify a consistent number of novel short RNAs expressed from miRNA loci in SET2 cells. It is worth notice that RNA discovery results were produced using only read aligning exactly to hairpin loci in order to minimize artifacts. At variance with other studies, we mapped

sequence reads to extended hairpin loci, ie the hairpin precursor genomic regions plus 30 nucleotides upstream and downstream. This allowed us to uncover novel short RNAs derived from extended hairpin loci transcription and canonical or non-canonical processing. More specifically, we discovered 78 novel miRNAs expressed from known hairpin precursors. These are all new miRNAs*, 11 of which are expressed in SET2 cells over the median value observed for the group of known miRNAs. These data confirm recent findings about the importance of miRNAs*¹⁸. Considering known and new miRNAs together, we took into account expression behavior of pairs of miRNAs derived from 5 and 3' strands of the same hairpin (miRNA/miRNA*, so called "sister miRNAs"). Sister miRNAs have different sequences and target different sets of coding RNAs. We showed that for about one half of hairpins both sister miRNAs are expressed concurrently; very likely, both contribute to target repression. Moreover, slightly less than one quarter of concurrently detected sister miRNA pairs were expressed at the same or comparable level. No strand prevalence was observed. These results further support theories about the not deterministic nature of strand selection bias, that appears to be regulated in cell-, tissue- and condition-specific way (Biasiolo et al., 2011; manuscript submitted). Also, our findings are compatible with the two-steps cleavage of hairpin RNA by Dicer¹⁶; bidirectional binding of processed dsRNAs by Dicer may indeed results in directional presentation of double strand miRNA duplex to Argonaute, influencing the strand selection bias.

Interestingly, we found short expressed RNAs derived from regions of extended hairpins outside known and new miRNAs. These are members of a novel class of miRNA-related RNAs, called micro-RNA offset RNAs (moRNAs)²⁵ recently identified by massive short RNAseq. moRNAs are currently defined as ~20 nt long RNAs that originate predominantly from the 5' arm of pre-miRNAs, with a biogenesis linked to that of miRNAs but not necessarily interdependent. These studies also indicated that moRNAs are generally included in the miRNA hairpin precursor, and while the moRNA usually overlaps the miRNA position by a few nucleotides, sometimes it overhangs the miRNA hairpin. In present study we identified 58 moRNAs expressed from 56 hairpins at moderate to high level. In

particular hsa-5'-moR-103a-2, hsa-5'-moR-106b, hsa-5'-moR-19b-1 and hsa-5'-moR-16-1 were highly expressed in SET2 cells. It was also of interest that 5 of the 16 most expressed moRNAs (Table 4) were associated with the 17-92 (miR-19b-1, miR-20a, miR-92a, miR-19a) and the 106-25 (miR-106b) cluster, as discussed above for miRNAs. Of note, some of the highly expressed moRNAs were unique and did not perfectly overlap the most expressed miRNAs from the same cluster, suggesting that activation of the cluster might result in global up-regulation of the genes but also in a non-balanced ratio between miRNAs and moRNAs (Table S10).

We observed a prevalence of moRNAs derived from the 5' arm of hairpin precursors, similar to previous reports, and showed that the prevalence of 5' moRNA is independent from miRNA strand selection bias. We found moRNAs longer than previously reported, with an average length of 20.2 nt, and a maximum of 28. A large majority (84%) of 5' moRNAs overlapped the 5' miRNA hairpin precursor boundary. Since moRNAs span the canonical Drosha cutting site, non-canonical Drosha cleavage might conceivably be responsible for moRNA production. Moreover, we observed that slightly less than one half of moRNAs overlapped the nearby expressed mature miRNA of a few nt; in these cases, moRNA and miRNA expression from the same precursor were mutually exclusive. Among the 9 most expressed moRNAs, five overlapped the corresponding miRNA. Interestingly, the expression ratio between 5' moRNA and miRNA pairs was much variable: the hsa-miR-103a was expressed almost twice the hsa-5'-moR-103a-2, the most expressed moRNA, and the two expression values were of the same degree of magnitude (2,809 versus 1,098, respectively). A similar situation was observed for hsa-mir-19b-1 and hsa-mir-16-1. Conversely, other moRNAs that are produced from the same hairpin of miRNAs were expressed at definitely lower levels: expression of hsa-miR-21 was about 6,000 times higher than the hsa-5'-moR-21. The observation that moRNA sequences are as conserved as miRNAs gives additional, although indirect, support to the hypothesis that moRNAs plays a defined biological role. Evidence regarding possible functions of moRNAs is still fragmentary²⁵; one hypothesis claims that moRNAs might guide RISC to complementary target mRNAs as miRNAs do³⁴.

Nevertheless, the fact that moRNAs are nuclear enriched may support the alternative hypothesis that moRNAs intervene specifically in nuclear processes as other nuclear short and long RNAs do. Therefore, moRNAs can be viewed as a new class of regulators whose qualitative and/or quantitative abnormalities might impact on human diseases. In this view, the discovery of expressed moRNAs in SET2 cells is intriguing.

In summary, we have extensively characterized the profile of short RNAs expressed in SET2 cells and provided proof-of concept that modulation of mature miRNA expression may be obtained via inhibition of JAK2. It must be stressed, however, that SET2 cells are not a physiologic model of MPN, so any novel information should be validated in primary MPN cells. It will be key to understand how the interplay of known and new miRNAs, isomiRs, and moRNAs contributes to the abnormal regulation of cell proliferation that characterizes MPN cells and whether and how drugs affect this complex system of regulators. Finally, results regarding isomiRs and moRNA expression fit well in the “RNA in pieces” phenomenon³⁵: many transcripts undergo post-transcriptional cleavage to release specific, functionally independent, fragments expanding the spectrum of regulatory RNAs produced by the human genome and from single loci.

ACKNOWLEDGEMENTS

The study was funded by a grant from Associazione Italiana per la Ricerca sul Cancro (AIRC, Milano) “Special Program Molecular Clinical Oncology 5x1000” to AGIMM (AIRC- Gruppo Italiano Malattie Mieloproliferative), project number 1005. A detailed description of the AGIMM project is available at <http://www.progettoagimm.it>. AMV was also supported by AIRC project number 9034 and MIUR-20084XBENM_002. SB was also supported by Fondazione Cassa di Risparmio di Padova e Rovigo and University of Padova. R.N. is the recipient of a fellowship from “Collegio Ghislieri” Foundation.

AUTHORSHIP CONTRIBUTIONS

S.B., designed research, analyzed data, interpreted results, and contributed to manuscript writing; A.B., designed the computational pipeline with S.B., implemented it, analyzed data and contributed to manuscript writing; M.B., prepared target predictions and contributed to manuscript writing; P.G., performed research, interpreted results, and contributed to manuscript writing; F.B., performed research; R.N. performed research; R.M. performed and interpreted array expression experiments; A.M.V, designed research, analyzed data, interpreted results, and contributed to manuscript writing.

DISCLOSURE OF CONFLICTS OF INTEREST

All the authors state they have no relevant conflict-of-interest to disclose.

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TABLES

Table 1. Twenty-one miRNAs highly expressed account for 70% of total known miRNAs expression in SET2 cells.

miRNA	Expression level	Cumulative % of total known miRNAs expression
hsa-miR-21	2268333	12
hsa-miR-148a	1737364	21
hsa-miR-146b-5p	1632959	29
hsa-miR-101	1293074	36
hsa-miR-142-3p	1037658	41
hsa-miR-19b	1025647	46
hsa-miR-378	561770	49
hsa-miR-92a	538422	52
hsa-miR-191	416870	54
hsa-miR-425	391247	56
hsa-miR-126	349719	58
hsa-miR-17	314388	59
hsa-miR-181b	310800	61
hsa-miR-181a	301902	63
hsa-miR-93	223220	64
hsa-miR-126*	216687	65
hsa-miR-30e	201602	66
hsa-miR-99b	200438	67
hsa-let-7f	196921	68
hsa-miR-25	191693	69
hsa-miR-20a	186436	70

Table 2. Name and predicted sequence of 15 new miRNAs with read count greater than 500. Seven new miRNAs associated to read counts over 10^3 are shown in bold.

New miRNA	Sequence	Expression
hsa-miR-1307*	CTCGACCGGACCTCGACCGGCTCGT	72670
hsa-miR-376a-2*	GGTAGATTTTCCTTCTATGGTTA	14296
hsa-miR-382*	CGAATCATTACGGACAACACTTTTT	8296
hsa-miR-539*	AATCATACAAGGACAATTTCTTTTTGA	3332
hsa-miR-181b-1*	CTCACTGAACAATGAATGCAACT	1542
hsa-miR-561*	ATCAAGGATCTTAAACTTTGCC	1315
hsa-let-7c*	CTGTACAACCTTCTAGCTTTCT	1195
hsa-miR-652*	ACAACCCTAGGAGAGGGTGCCATTCA	982
hsa-miR-301a*	GCTCTGACTTTATTGCACTACT	880
hsa-miR-487a*	GTGGTTATCCCTGCTGTGTTGG	823
hsa-miR-370*	AAGCCAGGTCACGTCTCTGCAGTTACAC	624
hsa-miR-412*	TGGTCGACCAGTTGGAAAAGTAAT	578
hsa-miR-376c*	GTGGATATTCCTTCTATGTTTAT	568
hsa-miR-381*	AAGCGAGGTTGCCCTTTGTATATTC	567
hsa-miR-376b*	GTGGATATTCCTTCTATGTTTA	532

Table 3. Number of expressed miRNAs respective to number of known miRNAs, per hairpin (A). Percentages of hairpins associated to concurrently expressed miRNA/miRNA* pairs, according to different thresholds on expression level (B).

A

# known miRNAs	Hairpins	# of expressed					
		2		1		0	
		#	%	#	%	#	%
2	478	189	39.54	102	21.3	187	39.1
1	943	71	7.53	175	18.6	697	73.9
Hairpins	1421	260	18.30	277	19.5	884	62.2

B

Expression of at least	Hairpins with at least one miRNA expressed	Hairpins with two miRNAs expressed	% of expressed hairpins with two miRNAs
10	522	250	47.9
10 ²	331	175	52.9
10 ³	229	94	41.0
10 ⁴	125	15	12.0

Table 4. Name and predicted sequence of 16 new moRNAs with read count greater than 100. Four moRNAs associated to read counts over 500 are shown in bold.

moRNA	Sequence	Expression
hsa-5'-moR-103a-2	GAGCTGCGTCTTTGTGCTTTC	1098
hsa-5'-moR-106b	CCGCTCCAGCCCTGCCGGGGC	918
hsa-5'-moR-19b-1	TGTTACTGAACACTGTTCTATGGTT	700
hsa-5'-moR-16-1	TAGCAATGTCAGCAGTGCCT	506
hsa-5'-moR-20a	TGATGTGACAGCTTCTGTAGCAC	418
hsa-5'-moR-21	CATGGCTGTACCACCTTGTCGGG	345
hsa-5'-moR-27a	CTGTGCCTGGCCTGAGGAGC	306
hsa-5'-moR-125a	ACCATGTTGCCAGTCTCTAGG	300
hsa-5'-moR-377	CCGTGCTGATGTTTGACCCTTGAGCA	273
hsa-3'-moR-103a-2	AAGAACCAAGAATGGGCTGCC	206
hsa-5'-moR-941-3	CACCCGGCTGTGTGCACATGTGC	155
hsa-5'-moR-361	TTTTCTGGGATTTGGGAGC	154
hsa-5'-moR-92a-1	AACTCAAACCCCTTCTACAC	125
hsa-5'-moR-19a	TTTGTTTGCAGTCTCTGTT	119
hsa-5'-moR-7-1	GGATGTTGGCCTAGTTCTGTG	106
hsa-5'-moR-98	GGATTCTGCTCATGCCAGGG	102

FIGURE LEGENDS

Figure 1. The majority of miRNAs expressed in SET2 cells are mixtures of different isomiRs. A) Distribution of number of isomiRs per miRNA in the top 25% most expressed miRNAs and in the remaining more weakly expressed miRNAs. B) Contribution of the major isomiR per miRNA to the total count, for classes of highly expressed miRNAs composed of one to four isomiRs.

Figure 2. Relative isomiRs contribution to the total read count of 21 most expressed miRNAs in SET2 cells. The total read count belonging to sequences not passing the stringent threshold for being considered as isomiRs is indicated in the white rectangle.

Figure 3. Examples of new miRNAs discovered. Plots show the number of reads per nt position mapping in extended hairpin loci for hsa-mir-1307, hsa-mir-376a-2, hsa-mir-382 and hsa-mir-539, expressing both known and new miRNAs. Known miRNA position relative to the extended hairpin is indicated in the plot. In the upper part of panel, the sequence of mature miRNAs is shown (known miRNA in red, new miRNA in pink).

Figure 4. Two miRNAs and two moRNAs may be produced by transcription and processing of a single miRNA locus. The plots show expression levels of miRNAs, moRNAs and loops from 15 hairpins corresponding to most expressed moRNAs.

Figure 5. Selected examples of moRNA expression and sequence. Plots show the number of reads per nt position mapping in extended hairpin loci expressing miRNAs and moRNAs.

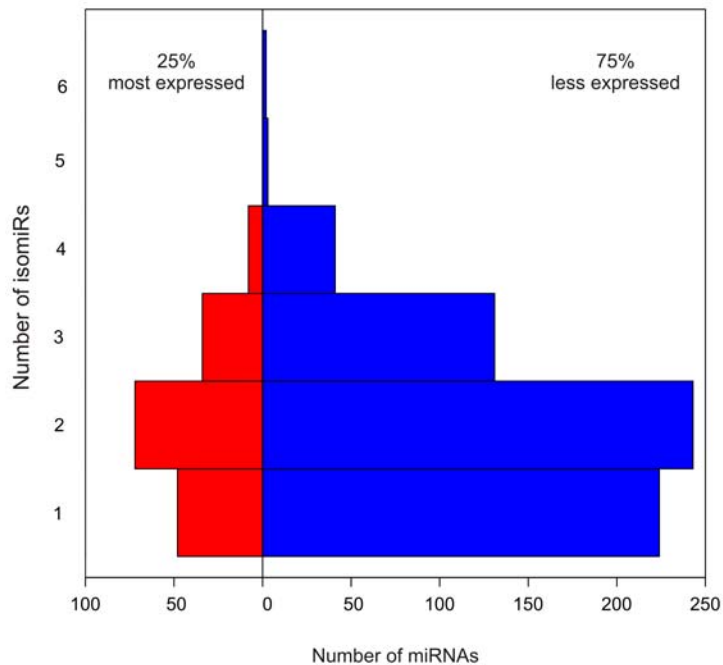
Figure 6. 5' moRNAs position relative to miRNA hairpin precursor (A) and to the mature miRNA expressed from the same hairpin arm (B). The number of expressed moRNA falling in each category is indicated close to the corresponding example.

Figure 7. Modulation of miRNAs by INC424/Ruxolitinib treatment of SET2 cells. Cells were exposed to INC414/Ruxolitinib at 160, 80 and 1,600 nM for 3 and 6 h, then processed for miRNA expression analysis by microarray. The concentration of 160 nM was selected as the IC50 concentration in a proliferation assay of SET2 cells (not shown). INC424/Ruxolitinib caused dose-dependent reduction of the level of phosphorylated STAT5 (measured by FACS) and Pim-1 mRNA (measured by quantitative real-time PCR), indicating effective inhibition of JAK/STAT signaling (top panels). In lower panels,

examples of INC424/Ruxolitinib induced miRNA level modulation, as measured using miRNA array, are shown. The levels of hsa-miR-92a-1* and hsa-miR-330-5p dose-dependently decreased, while those of has-miR-let-7c increased compared with untreated control cells. Dose effects were statistically significant ($P < 0.05$).

A

Distribution of number of isomiRs per miRNA



B

Major isomiR contribution to total count

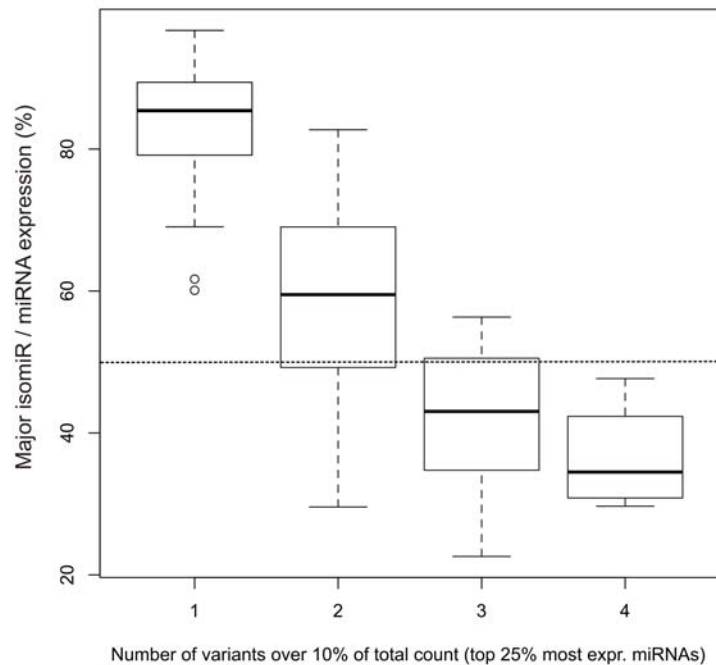


Figure 1

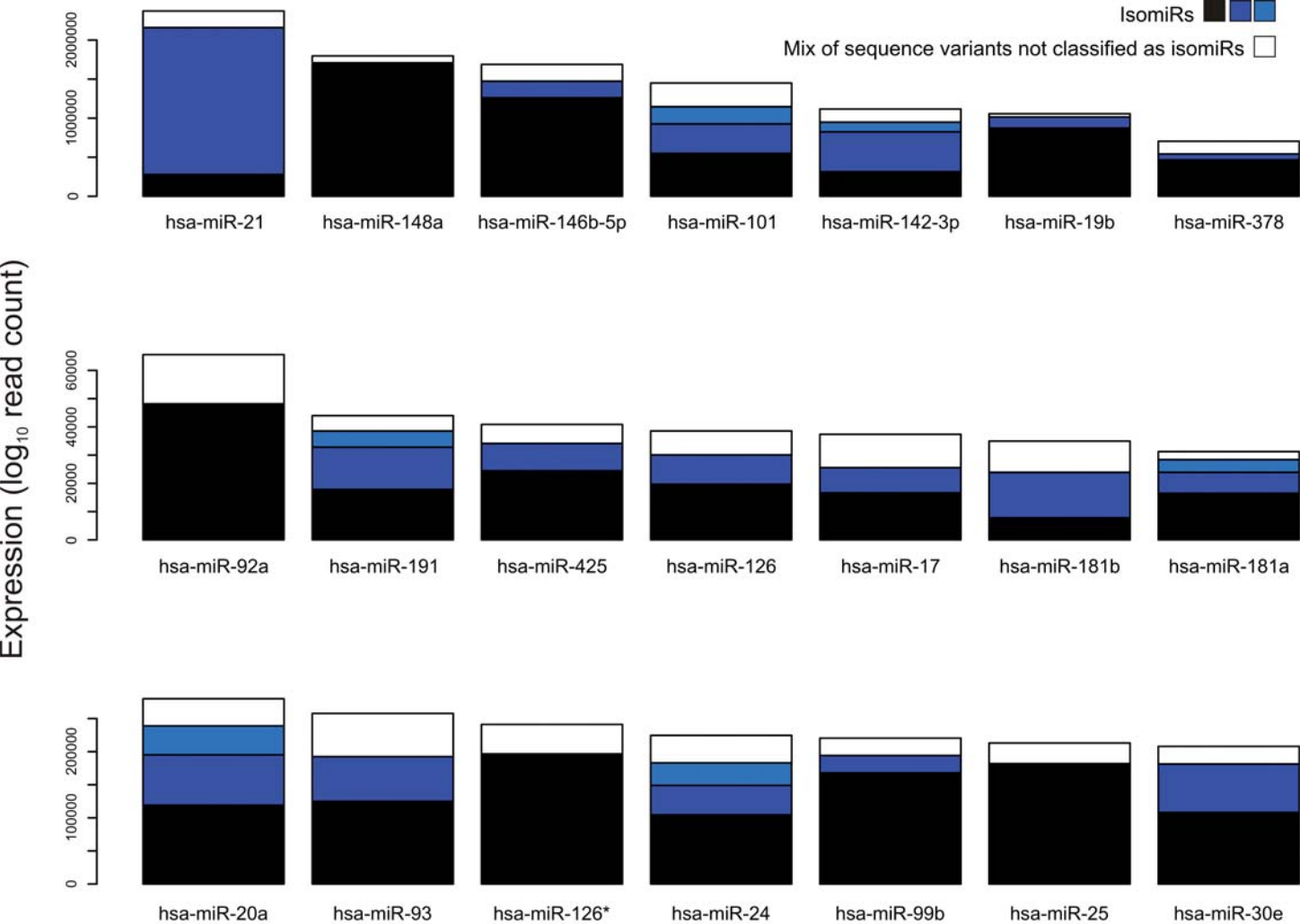


Figure 2

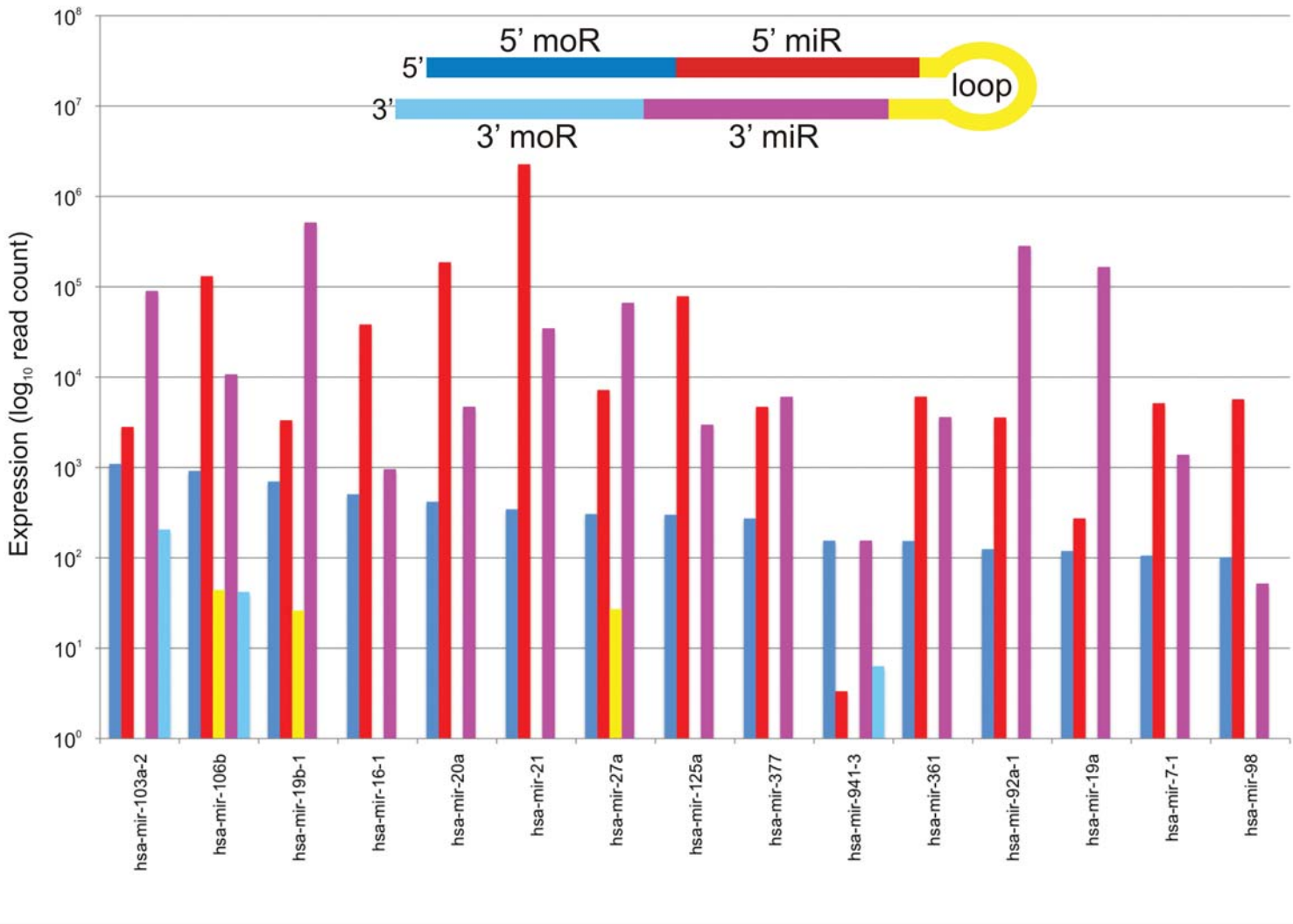


Figure 4

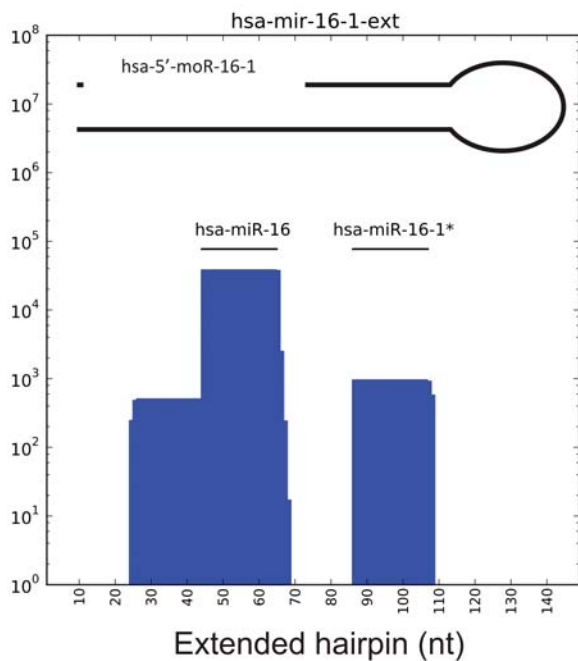
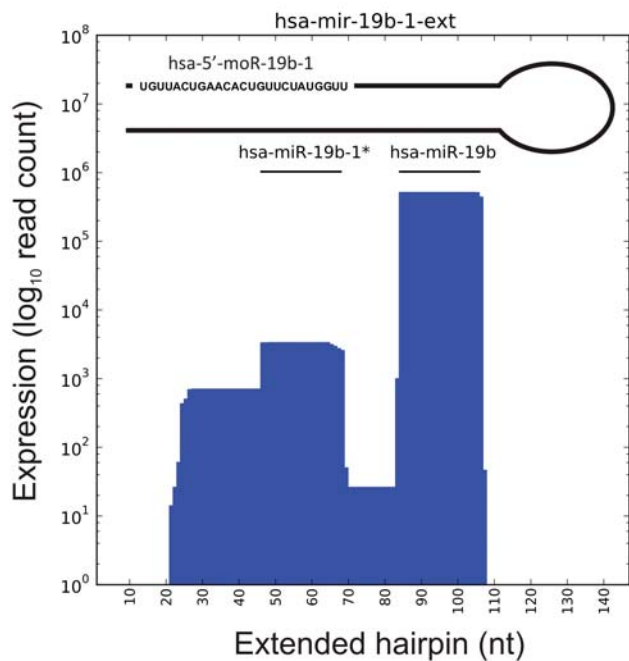
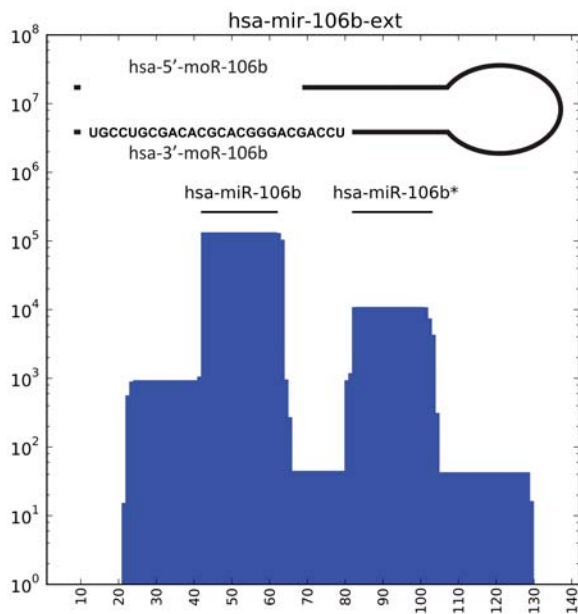
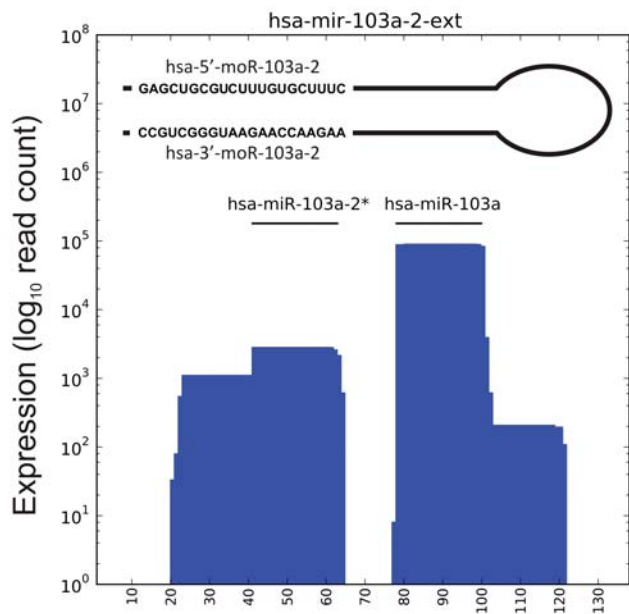


Figure 5

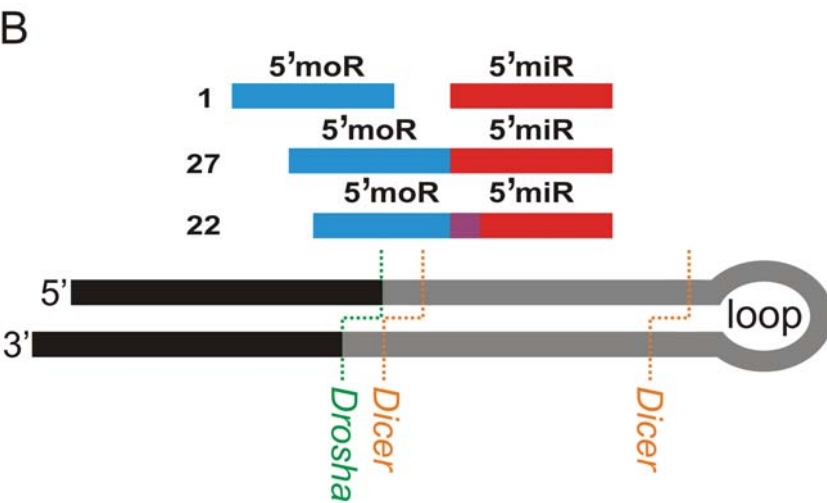
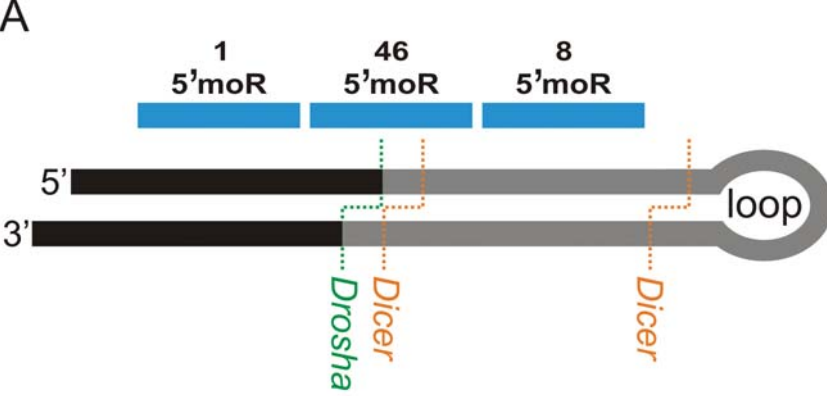


Figure 6

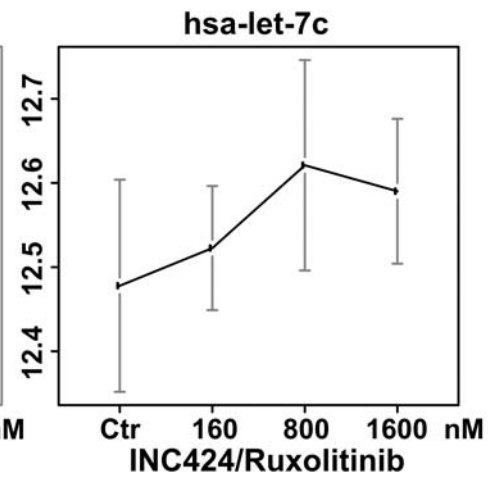
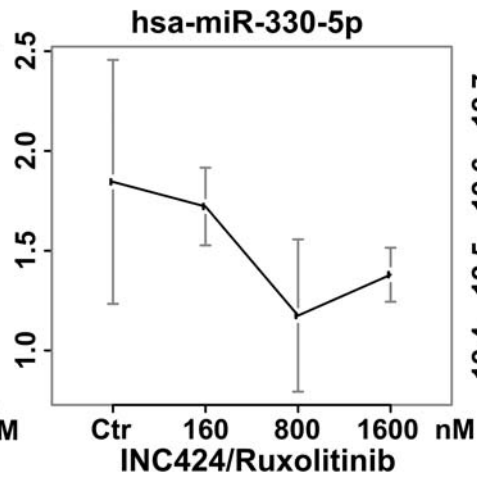
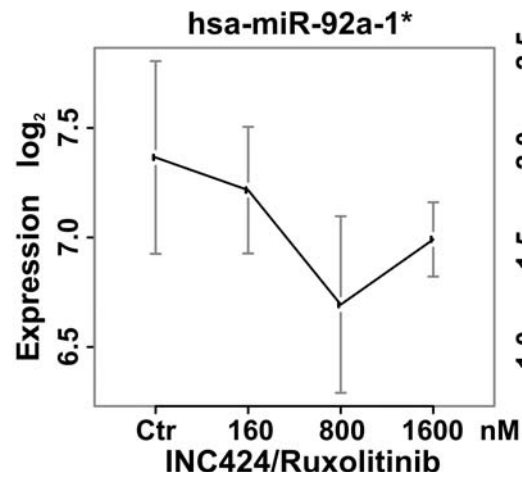
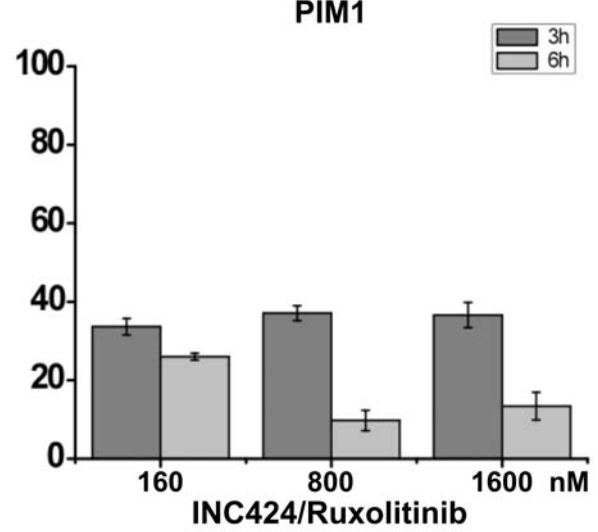
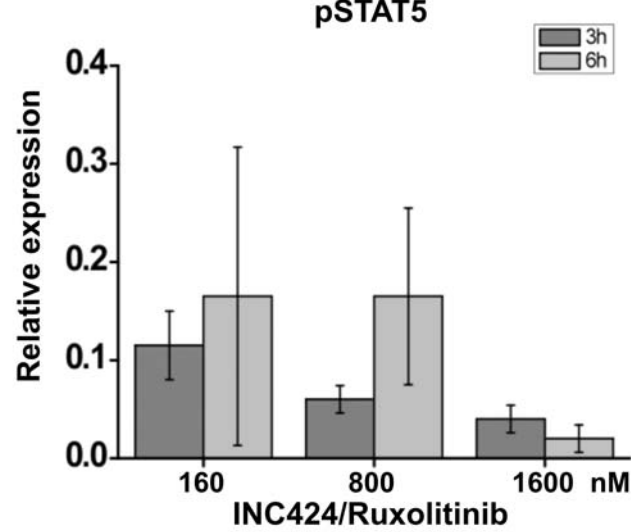


Figure 7