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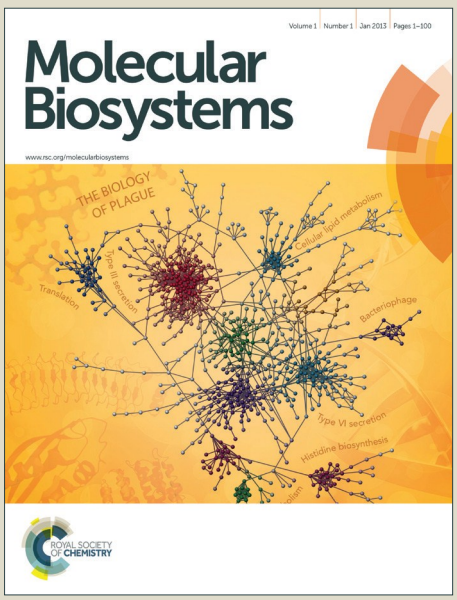

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ABSTRACT

Knockdown of single or multiple gene targets by RNA interference (RNAi) is necessary to overcome escape mutants or isoform redundancy. It is also necessary to use multiple RNAi reagents to knockdown multiple targets. It is also desirable to express a transgene or positive regulatory elements and inhibit a target gene in a coordinated fashion. This study reports a flexible multiplexed RNAi and transgene platform using endogenous intronic primary microRNAs (pri-miRNAs) as a scaffold located in green fluorescent protein (GFP) as a model for any functional transgene. The multiplexed intronic miRNA - GFP transgene platform was designed to co-express multiple small RNAs within the polycistronic cluster from a Pol II promoter at more moderate levels to reduce potential vector toxicity. The native intronic miRNAs are co-transcribed with a precursor GFP mRNA as a single transcript and presumably cleaved out of the precursor-(pre) mRNA by the RNA splicing machinery, spliceosome. The spliced intron with miRNA hairpins will be further processed into mature miRNAs or small interfering RNAs (siRNAs) capable of triggering RNAi effects, while the ligated exons become a mature messenger RNA for the translation of functional GFP protein. Data show that this approach led to robust RNAi-mediated silencing of multiple Renilla Luciferase (*R-Luc*)-tagged target genes and coordinated expression of functional GFP from a single transcript in transiently transfected HeLa cells. The results demonstrated that this design facilitates the coordinated expression of all mature miRNAs either as individual miRNAs or as multiple miRNAs and the associated protein. The data suggest that, it is possible to simultaneously deliver multiple negative (miRNA or shRNA) and positive (transgene) regulatory elements. Because many cellular processes require simultaneous repression and activation of downstream pathways, this approach offers a platform technology to achieve that dual manipulation efficiently. In conclusion, the current platform technology offers a miRNA/shRNA scaffold for the expression of combinations of native or synthetic intronic miRNAs as singletons or polycistrons for combinatorial multiplexed RNAi silencing or RNA-based gene therapy applications.

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INTRODUCTION

RNAi technology has become a powerful tool in reverse genetics and potentially as therapeutics. For example, the ability of RNAi to inhibit several viral [1], oncogenic, or several dominantly inherited metabolic and neurodegenerative genes have been shown in the literature [2]. Recent studies highlight the latest discovery of miRNAs involved in disease imitation an development including carcinogenesis as well as the potential applications of miRNA regulations in cancer treatment. Several studies have demonstrated the feasibility of restoring tumor suppressive miRNAs and targeting oncogenic miRNAs for cancer therapy using *in vivo* model systems using naturally occurring miRNAs expressed from viral vector systems[3]. It is estimated that more than 1000 miRNAs are exist in human and that 30% of the human genome is under miRNA regulation, one miRNA being able to modulate post-transcriptionally hundreds of downstream genes. Therfore, miRNAs have potential both as drug targets for miRA inhibition therapies and as drugs for miRNA replacement therapies [3]. However, considering the high sequence specificity of small interfering RNAs (siRNAs) and high variability of RNAi silencing by each siRNAs, the efficacy of siRNAs may be limited due to the target site accessibility or minor changes in the target [4-8] sequence, and even a single-point mutation is sufficient to overcome RNAi-mediated inhibition [2]. One approach to cope with this problem is using multiple siRNAs to simultaneously target multiple genes viral or host genes that are involved in the viral life cycle as well as multiple sites on the same gene. Furthermore, a multiplexed RNAi approach could be used for silencing multiple isoforms of a gene product where it is vital to address the issue of isoform redundancy.

Several reports have shown simultaneous silencing of multiple-gene expression in cultured cells by expressing multi-cistronic shRNAs from single promoters [9-20] or multiple shRNAs from individual transcription cassettes [16,17,19,21-29]. Additionally, a dual functioning aptamer-mediated cell type-specific delivery of combinatorial RNAi reagents also has been developed. For example, a number of studies have shown that HIV-neutralizing aptamers specific to either the CD4 receptor [30] or HIV-1 glycoprotein gp120 [31-33] have been used for targeting delivery of multiple anti-HIV siRNAs *in vitro* as well as in mice.

However, tandemly arranged transcription cassettes and loss of marker gene activity due to the processing of shRNAs, plagued these designs. Furthermore, when the same promoter is repeated in retroviral or lentiviral vectors, high frequency recombination occurs [17]. Another approach is to express multiple shRNAs from a single polycistronic shRNA transcript, such as a natural miRNA cluster that can be expressed from an RNA polymerase II promoter (RNA pol II). This strategy is

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of particular interest for antiviral purposes because miRNA-like transcripts were shown to be more effective in RNAi-mediated silencing than regular shRNAs [4,34]. Use of a RNA pol II promoter will permit more natural and regulated expression of shRNA transgene, thereby reducing the risk of toxicity due to oversaturation of the RNAi machinery [35,36]. For the past several years, evidence has accumulated that vector-mediated robust RNAi can trigger severe toxicity in small and large animals, from cytotoxicity and accelerated tumorigenesis to organ failure and death[36]. The recurring notions in these studies that a critical parameter is the strength of RNAi expression and that Exportin-5 and the Argonaute proteins are rate-limiting mammalian RNAi, strongly imply dose-dependent saturation of the endogenous miRNA pathway as one of the underlying mechanisms[35-37]. Additionally, some of these shRNAs are either expressed from RNA pol III promoters which naturally can only synthesize short RNAs, or from RNA pol II promoters without utilizing a natural miRNA platform and intron sequences where the processing of shRNAs could be a rate-limiting factor. Some reports have shown single hairpins expressed within intronic sequence [9], but not as multiple hairpin clusters for multi gene or site targeting. It is highly desirable to have a system that uses native miRNA pathway as much as possible so that it can enter the natural RNAi processing machinery. At the same time, the system should be a multi-component approach for targeting multiple genes that are expressed at equimolar concentrations on a single transcript and processed efficiently into functional siRNAs. Additional features such as coordinated expression of therapeutic or vaccine transgenes with the RNAi reagents will add additional features to the system that will have multi-faceted capability for manipulating the expression of pathogen or disease genes. When used in this context, both miRNAs and an exogenous transgene, such as a GFP reporter used in this study can be placed under the control of the same promoter for tracking successful transfection of target cells and identification of phenotype. In addition, designing such vector-derived intronic modified miRNA-based shRNA (miR-shRNA) approach in the context of a naturally occurring RNA pol II (*e.g.*, CMV) driven miRNA transcript as triggers of RNAi, increases the utility of this approach for conditional, cell type-specific RNAi [4,38-43]. Because natural intronic miRNA pathway is well coordinated by multiple intracellular regulatory systems, including RNA pol II transcription, RNA splicing, exosome digestion, and nonsense-mediated decay (NMD) processing [44,45], the gene silencing effects of intronic miRNAs are considered to be more natural and effective than the artificial shRNA hairpins [46].

The simultaneous expression of single or multiple intronic miRNAs with a transgene as a single transcript, and their ability to induce potent knockdown of their targets and functional expression of transgene from a single cassette enables simultaneous multiplexed gene knockdown and transgene

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expression in the same system. This should facilitate applications of simultaneous gene knockdown and corrective transgene expression in polygenic therapeutic targets, drug resistance genes, viral and host genes involved in viral life cycle, or oncogenes while overexpressing tumor suppressive genes. Indeed, a recent study showed the translational impact of a similar approach to conduct a Phase I trial using "bishRNA-furin/GMCSF DNA autologous tumor cell" vaccine in cancer [47].

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RESULTS

Identification, cloning, and functionally testing human intronic miRNAs. The goal was to identify, clone, and functionally test numerous native intronic miRNAs for their ability to silence their respective genes and preserve intron activity to express a functional transgene from the same transcript. This required identification of natural human intronic miRNA sequences from various databases (http://microrna.sanger.ac.uk/; www.genome.ucsc.edu; http://miRnamap.mbc.nctu.edu.tw) and literature [48,49]. This effort has led to the identification of a number of 18 human intronic miRNAs (**Table 1**) that fit the following criteria: (1) the intronic-miRNA sequences must have a human origin and be ubiquitous in various tissues; (2) they should be less than 1.2 kb in size containing the intron sequences for easy cloning; (3) they should have clearly identifiable intron sequences including the essential intron elements such as splice donor and acceptor sites and single branch points and well defined polypyrimidine track (PPT); and (4) they should be devoid of restriction enzyme sites that are being used for cloning into the expression vector. Functional testing of these constructs to induce RNAi-induced silencing of *R-Luc*-tagged target gene expression and subsequent functional GFP transgene translation is summarized in **Table 1**. Hypothetically, the intronic miRNA is co-transcribed with a precursor messenger RNA (pre-mRNA) by RNA polymerase II and cleaved out of the pre-mRNA by an RNA splicing machinery, spliceosome. The spliced intron with hairpin-like secondary structures is further processed into mature miRNAs capable of triggering RNAi effects, while the ligated exons become a mature messenger RNA (mRNA) for protein synthesis. However, because several (miR26b and miR-208a) constructs did not yield a functional GFP (*i.e.* no fluorescence) in transfected cells, implying that that miRNA biogenesis is not dependent on successful intron splicing or may not be prerequisite for RNAi activity. Alternatively, there could be a cryptic splicing site leading to successful exon splicing but results in nonfunctional GFP protein production.

Monitoring the miR-shRNA-induced RNAi-mediated gene silencing using a miR-shRNA-GFP expression and *R-Luc* reporter system. To monitor the RNAi activity of these intronic miR-shRNA vectors, we created a Luc-tagged miR-shRNA reporter system by cloning target sequences consisting of ~21-22 nt native miRNA target sequences into the 3'end of the Renilla (R) luciferase (Luc) gene of psiCHECK-2 dual luciferase reporter vector (Promega) (**Fig. 1**), and functionally tested their ability to silence the expression of *R-Luc*-tagged target sequences expressed from the dual-Luc-reporter vector (**Fig. 2**). Initiation of RNAi process by miR-shRNAs toward the Luc-tagged target gene both expressed episomally from the plasmid vectors in HeLa cells and resulted

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in cleavage and subsequent degradation of the fusion *R-Luc* mRNA and expression of functional GFP protein.

Individual intronic-miRNAs elicit functional GFP expression. Testing of multiple individual intronic miRNA-GFP constructs for their ability to promote correct intron excision and mRNA splicing showed robust and functional GFP expression in cells transfected with multiple expression vectors. Coupled expression of intronic miRNAs with GFP transgene could be seen as early as eight to 24 hrs. after transfection of cells (**Fig. 3**). Here, the GFP transgene provided immediate and direct evidence of correct mRNA splicing and translation of correctly folded functional protein which required correct intron excision and RNA splicing by native intronic-miRNAs. However, some of the constructs did not lead to functional GFP expression even though the sequences of their introns were confirmed (**Table 1**). This finding suggests that some sequences may contain alternative cryptic splice sites resulting in alternatively-spliced nonfunctional mRNA sequences. However, those failed to splice correctly and as a consequence did not produce functional GFP induced robust RNAi (**Table 1**). For example, constructs miR-208a and miR-26b did not result in functional GFP expression but promoted robust RNAi activity (**Table 1** and **Fig. 4**). Since intronic-miRNAs are within the GFP transgene, intron removal is necessary for correct mRNA splicing but this may not be required for miRNA processing and RNAi activity. This suggests that pre-miRNA processing by Drosha does not depend on correct intron excision and mRNA splicing, or alternatively, intron may be spliced at alternative splice site yielding unproductive or nonfunctional GFP protein production.

Intronic-single miRNAs co-expressed with reporter GFP gene lead to robust and specific RNAi activity. The functional assessment of these construct demonstrated not only the correct mRNA splicing and expression of functional GFP protein, but also led to efficient RNAi knockdown. The pre-miRNA was able to enter the natural RNAi biogenesis pathway leading to initiation of a robust and sequence specific RNAi activity which resulted in subsequent silencing of the cognate mRNA (**Fig. 3 and 4**). Whereas, pGI-GFP empty vector without the miRNA insert, and vectors with non-target specific irrelevant miRNA inserts, did not show any RNAi activity towards these targets indicating that silencing is sequence specific (**Fig. 3 and 4**). We found that 17 of the 18 intronic-miRNAs were able to trigger strong specific inhibition of their cognate targets **(Table 1** and **Fig. 4)**. Interestingly, two of the seven intron-miRNAs that showed high levels of silencing (miR-26b, 88% and miR-208a, ~77%) did not lead to expression of GFP, suggesting that intron removal is not necessary for RNAi or that alternative intron splicing is taking place (**Table 1 and Fig. 4**). In addition, miR-126, miR302a, miR302b, and miR302c with dual targeting

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properties, (*i.e.* both strands of the mature miRNA sequences promote RNAi activity) resulted in marked and specific silencing of their specific targets (**Fig. 4**). Unlike non-homologous miRNAs, *i.e.* empty intron without the miRNA insert, intron with a non-target specific irrelevant miRNA insert have no silencing effects of targeted genes. Interestingly, clones that did not produce functional GFP were able to induce robust RNAi activity (**Table 1** and **Fig. 4**). This finding suggests that intronic miRNAs entered the natural miRNA pathway resulting in successful processing of pre-miRNA hairpins by natural miRNA pathway that resulted in effective RNAi activity. A single transcript expressing individual or cluster intronic miRNAs specifically and effectively silenced their Luc-tagged target gene expression. In addition, intronic miR-126 with dual targeting capability (*i.e.* both the guide (miR-126-5') and passenger (miR-126-3') silenced the expression of their Luc-tagged cognate target sequences.

Intronic polycistronic miRNA 106 and 302 cluster co-expressed with GFP induces RNAi knockdown and functional GFP expression. The luciferase activity in the HeLa cells transfected with the Luc-tagged reporter and intronic-miR-106/93/25 cluster (**Fig. 5**) and miR-302b/302c/302a/302d/367 cluster induced robust and sequence specific knockdown of their cognate target sequences (**Fig. 6**). Dual targeting miR-302b-5' and miR-302b-3' silenced their target. However, dual targeting miR-302c-5' did not induce any RNAi activity while miR-302c-3' showed robust RNAi activity. Additionally, non-homologous miRNAs as well as empty vector (pGI-GFP) without the miRNA hairpin sequence showed no effect on these targets. Both miR106 and miR-302 cluster constructs were also tested for their ability to induce successful intron excision and mRNA splicing and subsequent translation of functional GFP protein. Monitoring of cells for the miR-302a and reporter gene expression revealed miR-302 expression assessed by qRT-PCR and reporter gene expression as early as eight hrs. after transfection of HeLa cells (**Fig. 7**). Functional GFP expression provided immediate and direct evidence of correct mRNA splicing and translation of correctly folded functional protein from the intronic multi-miRNA cassettes.

Replacement of cluster miRNA hairpins with different miRNA sequences. To demonstrate that the natural cluster miRNA sequences can be engineered to target other sequences than those targeted by the native miRNAs, miRNA hairpin sequences were systematically replaced with individual miRNAs that were shown to be functional when expressed as single miRNAs (**Fig. 8**). To do this, each of the individual miRNA sequences were systematically replaced with pre-miRNA sequences by site-directed mutagenesis and their ability to induce silencing of the *R-Luc*-tagged cognate target sequences were tested in HeLa cells. The modified miRNA used to replace individual miRNAs within the miR-302 cluster were human miR-26b, miR-126, and miR-423, and

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miR-7-1 (**Fig. 8A**). All engineered miRNAs retained their natural loop sequences since the natural miRNA loop sequences are required for efficient nuclear export of the pre-miRNAs [50]. The single-stranded flanks of polycistronic pri-miRNA clusters were also retained to assure efficient processing by Drosha. To demonstrate that each unit of the miRNA cluster is functional, single intronic-miR-shRNAs were also constructed using miRNA miR-26b, miR-126, miR-423, and miR-7-1 in which each member of the miRNA directed against their natural targets with full complementarity. Each construct was tested by co-transfecting HeLa cells with individual *R-Luc*-tagged target constructs. In a transient but constitutive expression manner, the constructs that carry one miRNA silenced their target, and consistent with the design, the construct that carried multiple miR-shRNAs (*e.g.* miR-302 cluster) against all targets silenced only their cognate targets (**Fig. 8B**) and the degree of silencing was comparable between the single and multiple hairpin constructs (**Fig. 8A)**. However, not all pre-miRNA sequences within the miR-302 miRNA cluster were amenable for replacement with another pre-miR-shRNA. Only miR-423 which was substituted with miR-302a induced robust and sequence specific RNAi activity comparable to pGI-423, the intronic single mi-shRNA control plasmid (**Fig. 8A**). Other miR-shRNAs (miR-302b and 302c) which were replaced with other functional single miRNAs including miR-26b, miR-126, and miR-7-1 failed to induce RNAi activity (**Fig. 8A**). However, each of the other four native miRNAs still retained their functionality except the one that had been replaced with other miR-shRNA (**Fig. 8B**). In all cases, robust GFP expression was seen in cells transfected with each of these miRNA-GFP vectors indicating that replacement of miRNAs with engineered single miRNAs (*e.g.* miR-423) with differing target specificity did not affect the intron function and subsequent GFP mRNA splicing and translation. Since not every miRNA (*e.g.* miR-302b to miR-26b and miR-302c to miR-126 or miR-302d to miR-7-1) substitution induce RNAi activity, the single stranded flanking regions of the artificially introduced miRNA sequences may be sensitive to Drosha processing warranting further studies.

miR-302a expression is detectable by qRT-PCR. A lentiviral transgene was used to co-express single and multiple shRNA/miRNA hairpins with a reporter gene (red fluorescent protein, RFP) as a single transcript from the same expression cassette under a doxycycline inducible CMV promoter. Cells transduced with these vectors were assessed for the expression of miRNAs and reporter gene 48 and 96 hrs. after transduction with the viral vectors. All the viruses expressed the intended miRNA (*e.g.* individual or clusters of miR-302) within cells (**Fig. 7**). HEK293 cells naturally do not express miR-302.

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Number of miRNAs affect viral titer. Because the ultimate goal of developing this multicistronic multiplexed miRNA system was to express them from viral vectors, it was critical to test whether encoding multiple miRNAs would have any effect on the ability of viral packaging in HEK293 packaging cells lines which has functional miRNA microprocessor activity. To test this, HEK293T cells were transfected with vectors encoding single or multiple miRNAs derived from the miR-302 cluster alongside the helper plasmids and viral titer was determined 48 hrs. after transfection.

During the packaging of the viral particles, it was observed that lentiviral transgene expressing anywhere from single (e.g. miR-302b) to as many as five miRNA hairpins (miR-302b; miR-302b-302c; miR-302b-302c-302a; miR-302b-302c-302a-302d; and miR-302b-302c-302a-302d-367) resulted in significant reduction of packaged viral particles. For example, vectors encoding five miRNAs resulted in only $6x10^4$ (transduction unit (TU)/mL) packaged viral particles; whereas, viral transgene containing four miRNAs produced $1.75x10^6$ TU/mL viral particles; viral transgene containing three miRNAs produced $2.4x10^6$ TU/mL viral particles; viral transgene containing two miRNAs produced $4.25x10^6$ TU/mL viral particles; and viral transgene containing one miRNA produced $5.5x10^6$ TU/mL viral particles (**Fig. 9**). There was an inverse correlation between viral titer and the number of miRNAs encoded by the viral transgene vector ($R^2 = 0.986$). This suggests that proteins involved in miRNA biogenesis pathway such as Drosha/Pasha or others might have caused the reduced packaged viral titer due to the removal of these miRNAs from the transcript.

Drosha knockdown interferes with miRNA microprocessing but not the spliceosome function. Expression of multiple miRNAs in HEK293T packaging cells line are expected to negatively impact transcription of full-length viral RNA genome (miRNA-reporter gene) during packaging because of the endogenous microprocessor protein activity within the packaging cell line. To test whether inhibition of Drosha or Pasha activity have an impact in miRNA expression levels during packaging, a doxycycline inducible Drosha (and Pasha) knockdown HEK293T cell lines were established. These cell lines express Drosha or Pasha specific shRNAs. The cell line were generated by infecting HEK293T cells with a vector that expressed anti-Drosha or Pasha shRNAs under the control of a doxycycline inducible CMV promoter. Cells were infected with viral vectors that expresses anti-Drosha or Pasha shRNAs at MOI of 1, selected with puromycin (1 mg/mL) for 6 days. Selected cells were then induced by doxycycline (1 mg/mL) for 48 or 96 hrs. and were used for transfection with plasmid vectors that encoded miRNA hairpins either as single miRNA or multiples of miRNAs. The expression of red fluorescent protein (RFP) was assessed by fluorescent microscopy; whereas, levels of mature miRNAs were assessed by qRT-PCR. As an example, data (**Fig. 10**) showed that upon induction of Drosha knockdown, mature miR-302a production was

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reduced by 3-5 Ct at 48 and 96 hrs. after the beginning of induction of shRNA expression. Drosha knockdown did not affect the RFP expression although it was expressed from the same transcript. Because the mature miRNA levels are relatively low when Drosha activity is suppressed compared with empty vector (no Drosha suppression), implying that during packaging there will be more intact viral genome RNA with multiple miRNAs or shRNAs will be available for packaging resulting in higher viral titer.

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DISCUSSION

This study reports a flexible multiplexed miRNA/shRNA based gene suppressor and transgene overexpression platform for coordinated expression of intronic single or multi miRNA/shRNAs against single or multiple target genes, and a functional transgene expression from a single transcription cassette driven by a constitutive RNA pol II promoter. This flexible platform permits expression of multiple miR-shRNAs with their natural or artificial intron sequences imbedded in the open reading frame of a transgene or a reporter gene in a variety of formats. In this approach, efficient processing of miR-shRNA is essential for RNAi activity while correct splicing of the mRNA is necessary for correct transgene translation and function. Functional evaluation of 18 multiple intronic miRNA-GFP constructs for their ability for correct intron splicing by virtue of monitoring functional GFP expression and miRNA-mediated silencing of *R-Luc* tagged target gene expression showed that although almost all miRNAs exerted robust RNAi activity, two out of 18 constructs did not produce functional GFP in cells transfected with these vectors (**Table 1**). Data from this study suggest that intron splicing is not prerequisite for miRNA function.

The lack of expression of GFP did not affect RNAi knockdown of target genes (**Table 1**) suggesting that proper splicing of GFP is not required for proper miRNA processing in the nucleus by Drosha. However, proper splicing of GFP does not imply spliceosomal excision of intronic miRNAs, nor does Drosha processing of an intronic miRNA imply proper splicing of GFP. Several scenarios can be proposed to explain the failure of the two out of 18 constructs to produce functional GFP including cryptic and nonproductive splice sites, mutations in the splice junctions, as well as an antisense-mediated steric inhibition of target expression, RNAi mediated inhibition or some other unknown mechanism. Although, the presence and functional activity of RNAi factors in the mammalian nucleus has been the subject of considerable debate; a recent report has demonstrated that RNAi factors are present and functionally active in human cell nuclei [51].

A number of natural miRNAs exist in clusters of multiple identical or different copies [52-54]. Some natural miRNA precursors sometimes are located as clusters in the introns of protein coding genes and transcribed with precursor mRNAs from the same gene, suggesting that miRNA precursors can be excised from introns without disrupting production of the mRNA [48]. Intronic miRNA expression is a common event in mammals because ~50% of mammalian miRNAs are encoded within the introns of protein-coding genes [55]. These miRNAs are transcribed by type-II RNA polymerases and excised by spliceosomes distinct from microprocessing to form mature miRNAs [56,57].

For instance, miR-302 family consists of highly homologous intronic miRNA members, which are transcribed together as a noncoding miRNA hairpin cluster containing miR-302b, miR-302c, miR-

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302a, miR-302d, and miR-367 in a 5'-to-3' direction [58]. They are expressed most abundantly in human embryonic stem (ES) cells and quickly decrease after cell differentiation and proliferation [58,59]. Several miRNAs (*e.g.* miR-302) expressed by a lentivirus as single and multiple miRNA hairpins tagged with RFP were detectable by qRT-PCR in HEK293T cells, which do not express miR-302 (**Fig. 7**). The ability to express multiple shRNAs or miRNA-derived shRNAs from a single transcript has been previously reported [4,39-43,60,61], and recent reports have shown that it can also be used to improve knockdown efficiency by expressing multiple miR-shRNA against the same target gene [20,62], and to promote multi-gene knockdown [20,62,63]. However, poor processing of shRNAs as well as loss of transgene activity due to the processing of shRNAs plagued these designs.

Most studies have used either the miR-30 or miR-155 backbone because these have been studied extensively. This study focused on the natural miR-106, miR-302 (**Table 1)** clusters and number of intronic-single miRNAs. Here the aim was to use primary miRNAs in their natural form with their own intronic sequence or with an artificial mini intronic sequence to develop a combinatorial RNAi approach with coordinated transgene expression capability. Individual or polycistronic pri-miRNA cassettes were expressed from a RNA pol II promoter. Data demonstrate robust and specific silencing of *R-Luc*-tagged miRNA targets and coordinated expression of functional GFP in mammalian cells transfected with these vectors encoding single or multiple miRNAs.

However, engineering these miRNAs to target different sequences posed a challenge. With the exception of miR-302a within miR-302 polycistron which was replaced with miR-423, all the other miRNAs including miR-26b, 126, and 7-1 failed to exert RNAi mediated inhibition of their cognate target (**Fig. 8A** and **B**).

All engineered pri-miRNAs had retained their natural single-stranded flanking sequences thought to be required for proper Drosha/Pasha processing. To prevent the introduction of foreign restriction enzyme recognition sequences, a site-directed mutagenesis method was used to alter the sequence of each miRNA without the cloning steps. Since every siRNA sequence will affect the folding of the miR-shRNA, this could affect the position of Drosha processing resulting in constructs that cannot lead to the production of non-functional mature miRNA or siRNAs. Therefore, careful positioning of the gene specific miR-shRNAs is a prerequisite for production of effective RNAi reagents. From the current data, only the miR-302a was amenable for replacement with other miRNAs, in this case miR-423.

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If engineering challenges can be overcome, use of natural intronic miRNAs to express gene specific shRNAs and a transgene of interest provide a powerful new strategy for modulation of gene expression *in vitro* and potentially *in vivo*. It was previously reported that due to the mimicry of natural miRNA pathway, Pol-II-directed intronic miRNA expression does not cause any cytotoxicity [59], whereas Pol-III-directed siRNAs induced non-specific mRNA degradation [64]. Since the system utilizes a natural platform, expressed shRNAs should enter the natural miRNA pathway resulting in greater activity while avoiding activating interferon related pathways or saturating the miRNA pathway [35]. Because the natural intronic miRNA biogenesis is naturally regulated, the gene silencing of intronic miRNA is considered to be the most effective, target specific, and safe approach. Recent studies suggested that oversaturation of miRNA biogenesis pathway, shared by the shRNA/miRNA pathways, may lead to cytotoxicity [35-37]. Recent evidence suggest that siRNA sequences inserted in miRNA sequences scaffold do not compete for transport and incorporation into RISC, while simple shRNAs or synthetic siRNAs result in competition with natural miRNAs [65]. It has been suggested that intronic pri-miRNAs are processed by spliceosome components; whereas, intergenic pri-miRNAs are processed by Drosha-like RNases in the nucleus yielding pre-miRNAs [66].

Coupled-expression of a transgene such as GFP from the same transcription unit with RNAi permits the use of natural miRNA platform to simultaneously promote RNAi induced knockdown of target genes and overexpress a transgene. Although GFP was used as a proof-of-concept transgene in this study which also allowed easy monitoring of the transfected cells, any other transgene including drug resistance genes, marker genes, transcriptional activators, or therapeutic genes could be used in place of GFP. The intronic miR-shRNA-transgene cassette is expressed in a natural context and regulatable fashion from constitutive, inducible, or tissues specific pol II promoters. This restricts RNAi and transgene expression in time and space, and should reduce non-specific effects due to their effect in non-targeted tissues.

The transgene “knockin” and RNAi “knockdown” dual functional platform provides new opportunities for multi-gene targeting and simultaneous transgene expression strategies. Simple in their architecture, such combinatorial constructs are capable of simultaneous expression of multiple miR-shRNAs and transgene(s). This could have utility in a wide variety of areas. For instance, in genetic function analysis, multi-gene constructs could be used to investigate combinatorial effects to determine the presence of functional redundancy, or the degree of synergistic or antagonistic interplay between molecular components. In addition, the ability to coordinate transgene expression with gene silencing renders the technology useful for therapeutic applications which

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may require the silencing of a faulty gene or allele followed by substitution with a wild-type or functional gene. From a practical perspective, single multi-expression constructs also possess several useful characteristics compared to multiple individual constructs used either in combination or successively. By ensuring all genetic elements are carried on the one construct ensures that gene expression and RNAi silencing occur with less variability across a transfected cellular population. With all transgenes and intronic miR-shRNAs expressed from the single promoter, the relative expression level of each gene and miR-shRNA should be more similar from cell-to-cell and experiment-to-experiment than when multiple constructs are used. Furthermore, the ability to affect multiple interventions simultaneously should accelerate the production of stable cell lines and other transgenic organisms by avoiding the need for multiple selections or crosses.

This multi-silencing construct can be used in investigations where knockdown of multiple genes is required. For instance, it can be used to investigate whether genes are in sequential or parallel pathways, and whether genes are performing redundant roles and acting synergistically or antagonistically. Compared with the gene knockout approach, this multiple knockdown approach can avoid having to carry out multiple crosses of individual knockout lines and therefore, can accelerate investigations of gene-gene interactions *in vivo*.

In fact, the translational utility of this approach has been demonstrated in a recent Phase I trial [47]. In that study, researchers used a plasmid encoding granulocyte-macrophage colony-stimulating factor (GMCSF) and two unique shRNAs targeting furin convertase, thereby regulating endogenous immunosuppressive transforming growth factors (TGF) B1 and B2 as an autologous tumor cell vaccine to evaluate the triad immunotherapeutic concept of concurrent autologous provision, immunostimulation, and inhibition of autologous whole cell component endogenous immunosuppression [47]. Study showed that the plasmid based vaccine was safe and elicited immune response correlating with prolonged survival.

In summary, this approach permits: (**i**) simultaneous gene knockdown and transgene expression (*e.g.* silence an oncogene and express a tumor suppressor gene or silence a mutant gene and deliver an RNAi resistant functional copy) of a single gene; (**ii**) simultaneous silencing of multiple genes (*e.g.* viral and host genes as well as multiple isoforms of the same gene products); (**iii**) vector based-vaccine therapy where delivery of a viral gene could be used to induce an antiviral cellular immunity, and RNAi component can be used to silence viral and /or host genes; (**iv**) multi-site targeting of a single gene for synergistic and potentially additive RNAi activity; (**v**) expression of dual-targeting shRNAs (both guide and passenger strands functions as triggers of RNAi) for

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targeting RNA viruses that have both positive and negative strand genomes in their life-cycle; (**vi**) multiple shRNAs targeting the same gene simultaneously reduces the time for selecting potent shRNAs from multiple individual shRNA designs; (**vii**) constitutive or regulatable expression from pol II promoters; and (**viii**) tissue specific expression from tissue specific promoters for site specific gene knockdown. Potentially, this technology could also be useful for any genetic manipulation strategies requiring multiple interventions and could benefit many applications including, polygenic therapies, and general genetic analysis.

Furthermore, findings from this study represent one of the first examples of incorporating combinations of naturally occurring intronic miRNA singletons or polycistrons based agents with a protein coding transgene such as GFP reporter gene within a single expression platform driven by a single RNA Pol II promoter. The study demonstrated the versatility of the single or multi-miRNA-GFP platform for expressing a variety of miRNA expression and examining their function using a luciferase reporter system.

The study also demonstrated that engineering some of the miRNAs to target any other desired gene mRNAs within the native miR-302 or any other polycistronic miRNA cluster is challenging. This requires a systematic and careful optimization of the new pre-miRNA or shRNA sequence intended to target other sequences such that they can be processed properly by the endogenous miRNA microprocessor machinery (Drosha/Pasha, *etc.*) yielding mature and functional RNAi reagents (miRNA or siRNA).

The study demonstrated that more miRNA expression is not always better but rather a balance between transgene expression, target specificity and selectivity and potency of RNAi-mediated target inhibition, and target choice is required for optimizing the combinatorial approach. Interestingly multiple miRNAs within the same cassette negatively affected packaging of these constructs versus the cassettes expressing single miRNA which resulted in the reduction of the viral titer (**Fig. 9**). Data showed that when expressed as singletons or multiples of each of the miRNA subunit of the miR-302 cluster, packaged viral titer was inversely correlated with the number of miRNA subunits (R^2 = 0.9857) (**Fig. 9**). The reduced packaging efficiency was especially pronounced when the transgene encoded more than two miRNAs. One could argue that this could also be due to saturation of the RNAi pathway; however, this is highly unlikely because the transduced HEK293T cells were infected at MOI of 1 and drug selected; thus, will only have a single lentiviral vector copy. Another possibility is that proteins involved in upstream miRNA

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biogenesis in the nucleus such as Drosha and Pasha could be processing the miRNA hairpins as the transcription summons.

Several precious studies suggested that a critical parameter is the strength of RNAi expression and that Exportin-5 and the Argonaute proteins are the rate-limiting mammalian RNAi, implying a dose-dependent saturation of the endogenous miRNA pathway as one of the underlying mechanisms[35-37]. Rossi *et al.* [27] found that expression of the anti-HIV RNAs could negatively impact transcription of the full-length viral RNA genome during packaging because the pHIV7-EGFP lentiviral vector is dependent on HIV-1 Rev for packaging. To address this, they used a plasmid that expressed an Ago2-targeting shRNA to minimize the RNAi activity in cells during packaging and found no advantage to downregulating Ago2. Thus, there could be an advantage to downregulating Drosha or Pasha or both because the mature miRNA levels are relatively low when Drosha activity is suppressed compared empty vector (no Drosha) (**Fig. 10**), implying that during packaging these should result in higher viral titer when multiple miRNAs are packaged. To address this issue, an inducible Drosha knockdown HEK293T cell line was generated and used to temporarily silence Drosha. Data demonstrated that reporter gene (RFP) was still expressed because they were processed by the spliceosome, but the miRNAs processing was interrupted as reflected in the reduction of mature miRNA levels measured by qRT-PCR (**Fig. 10**) suggesting the loss of RNAi activity. Thus, based on this limited data, it is proposed that Drosha could be one of the bottleneck in the production of functional shRNAs. Previous data supports this finding that suppression of miRNA silencing pathway results in improved HIV titer [67]. Nevertheless, additional studies are warranted to overcome this challenge by targeting the key components of the miRNA biogenesis pathway in addition to Drosha or Pasha or both simultaneously using the very technique proposed in the present case and assessing viral titers in engineered packaging cell lines. With the improvement in vector efficacy and strategies to increase the packaging efficiency with multiple miRNA/shRNA sequences, it is hoped to improve the current platform for multiple basic science and gene therapy applications.

Collectively, these factors should be carefully considered in designing an efficient RNA-based multiplexed and combinatorial gene repressor and overexpression systems as a platform technology for basic science and for gene therapy applications as evidenced by recent studies [17,27,47].

CONCLUSIONS

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miRNAs are noncoding RNAs with high tissue specificity and play a key role in the regulation of a broad spectrum of developmental, physiological, and pathological processes[68]. Various miRNAs have been described as oncogenes or tumor suppressors and many of them are used for diagnosis and as prognostic or predictive tools [68]. Emerging data have demonstrated the feasibility of restoring tumor suppressive miRNAs and targeting oncogenic miRNAs for cancer therapy using *in vivo* model systems [3]. However, future studies are warranted for the optimization of the miRNA and transgene expression and delivery systems to simultaneously deliver multiple negative (miRNA) and positive (transgene) regulatory elements. In addition, understanding and control of off-target effects of miRNA therapeutics are challenges for the future development.

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MATERIALS AND METHODS

Cell culture and transfection. HeLa cells were grown in Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10% fetal bovine serum (FBS), 100 U/mL penicillin, and 100 µg/mL streptomycin. Twenty-four hrs. before transfection, cells were plated in 96-well plates at cell density of 5000 cells per well in DMEM supplemented with 10% FBS without the antibiotics. On the day of transfection, 60 ng plasmid expressing intronic miR-shRNA-GFP constructs, 40 ng *R-Luc*-tagged miR-shRNA reporter plasmids or 100 nM Luc-siRNA targeted against firefly luciferase gene of the *R-Luc* reporter vector, and 0.3 µl Lipofectamine 2000 (Invitrogen) were combined in 34 µL OptiMem (Gibco-BRL). Cells were transfected following manufacturers protocol in a final volume of 100 µL per well. The FBS was added back 4 hrs. post-transfection. After 48 hrs., dual luciferase activities were determined by assaying the cell lysates with the Dual-Luciferase Assay kit (Promega) according to the manufacturer's protocol. The luciferase activity was expressed as the ratio of Renilla-luc activity to firefly-luc activity. This ratio was further normalized to the control, which was transfected with the empty pGI-GFP carrier vector.

Fluorescent microscopy. The HeLa cells transfected above were analyzed under a fluorescent microscope 24 hrs. after transfection for monitoring the GFP expression as described[69]. GFP fluorescence in HeLa cell monolayers was observed under illumination with the 360–400 nm spectrum of light that excites GFP fluorescence. Fluorescent images of cells were obtained under 10x or 20x magnification on a Nikon TE 300 microscope (Nikon Instruments, Lewisville, TX) using a DXC-970MD CCD camera (Sony Corporation, New York). The GFP fluorescence was determined visually.

PCR amplification of intronic-miRNAs from genomic DNA. Total HeLa genomic DNA was extracted from 5E6 cells using DNeasy Genomic DNA isolation kit (Qiagen) and the integrity of DNA was verified on a 0.8% agarose gel. Natural single intronic miRNAs (**Table 1**) and polycistronic intronic miRNA clusters miR-106 and miR-302 consisting of three and five hairpins respectively, were PCR amplified from genomic DNA of HeLa cells. Primers used for the PCR were complementary to the flanks of the splice donor (SD) and splice acceptor (SA) sites of each intron of the miRNAs. Forward and reverse primers contained SacI/SacII and BstEII restriction enzyme sites for cloning into GFP gene. The primers used to amplify intronic-microRNAs are shown in **Supplementary Table 1**. The PCR conditions were as follows: The intronic-miRNAs were amplified from 10 ng HeLa genomic DNA with Pfx DNA polymerase (Invitrogen) with 1 µM

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of each forward and reverse primers, 0.3 mM each dNTPs, 1.25 mM $MgSO_4$ in 1x PFX pol Buffer (Invitrogen). The PCR parameters were: 5 min. at 94°C for initial denaturation, 30 sec. at 94°C, 30 sec. at 54°C, and 2 min. at 68°C for 30 cycles, followed by 30 min. at 68°C for final extension.

Intronic miRNA expression vector construction. All intronic miRNAs, miR-shRNAs, and GFP reporter gene were cloned into the mammalian expression vector pGFP which was derived from pCMV-Tag 4 (Clontech). The intronic miRNA sequences were amplified by PCR from HeLa genomic DNA extracts using primers that were complementary to the 5'-3' splice junctions of each intron sequence. The PCR products were purified from an agarose gel, cut with appropriate restriction enzymes (Sac I/BstE II or SacII/BseE II, (NEB) and cloned into the GFP sequence after the start codon of plasmid pCMV-Tag 4 (Clontech). The 97-nt template was amplified with Vent R DNA polymerase (New England Biolabs) by 25 PCR cycles, each consisting of 30 sec. at 94°C, 30 sec. at 54°C, and 1 min at 75°C. Subsequently, 1 μL Taq DNA polymerase was added to the PCR product and incubated at 72°C for 10 min. The PCR products encoding intronic single- or multi-hairpin miRNA constructs were inserted into SacI/SacII and BsteII sites of the pGFP vector. As for the polycistronic intronic miR-106 and miR-302 consisting of three and five hairpins respectively, entire intron and hairpin sequences were amplified by PCR from HeLa genomic DNA extracts using primers flanking the splice donor (SD) and splice acceptor (SA) sites and cloned into the SacI and BstE II sites of the GFP expression vector as described above. All sequencing were performed on the subcloning part and when cell lines were generated (as a SNP or a mutation could affect the efficiency of the targeting).

***R-Luc*-tagged miR-shRNA reporter vectors.** The perfect match target sites for the human miRNAs were cloned into the 3'-UTR of the Renilla (R) luciferase (Luc) gene (XhoI/NotI sites) in the psiCHECK-2 vector (Promega) by the ligation of the appropriate synthetic double-stranded DNA oligonucleotide duplexes, that contained appropriate overhangs complementary to those generated by restriction enzyme digestion of the vector. All constructs were sequence-verified. The psiCHECK-2 vector is designed to provide quantitative and rapid assessment of RNAi activity. This vector enables monitoring of changes in expression of target gene fused to a reporter gene. *Renilla* luciferase is used as the primary reporter gene, and the gene of interest is cloned into a multiple cloning region located downstream of the *Renilla* translational stop codon while firefly luciferase is used as internal l control. The miRNA targets for knockdown of luciferase expression were engineered such that the complementarity between the miRNA guide strand and the target has perfect homology leading to RNAi-mediated target mRNA cleavage. Initiation of the RNAi process by miRNA or shRNA toward a gene of interest results in cleavage and subsequent

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degradation of the fusion mRNA. Measuring decreases in *Renilla* activity provides a convenient way of monitoring the RNAi effect. The psiCHECK-2 vector contains a second reporter gene, firefly luciferase, and is designed for normalization of *R-Luc* expression, achieving robust and reproducible results. The target sequences (sense strand) for human intronic miRNAs had perfect complementarity to the native miRNA sequences so targets would be effectively suppressed by endogenous RISC. The target sequences (sense strand) for human intronic miRNAs (shown in **Supplementary Table 1**) had perfect complementarity to the native miRNA sequences so targets would be effectively suppressed by endogenous RISC.

Modification of native miRNA-302 cluster hairpins by site-directed mutagenesis to introduce different miRNA sequences. Site-directed mutagenesis was used to alter each of the hairpin sequence within the miR-302 cluster using ExSite^{TM} PCR-based Site-Directed Mutagenesis kit with some modifications (Stratagene). PCR reactions were performed with 1 uM of each primer set, 145 ng of plasmid template, 1.5 mM $MgCl_2$, 0.2 mM each dNTPs, in 30 μL in 1x PCR reaction buffer (Stratagene). The PCR parameters were: 30 sec. at 95°C for initial denaturation, 30 sec. at 95°C, 1 min. at 55°C, and 5.50 min. at 68°C (1 min./kb) for 20 cycles with 30 min. at 68°C for final extension. 1 μL of Dpn I (10U/μL, NEB) was then added into the reactions and parental plasmid was digested at 37°C for 1 hr. Products were verified on a 0.8% agarose gel by EtBr staining. PCR reactions were diluted to 130 μL and ligated with T4 DNA polymerase in the presence of 1x T4 DNA ligase buffer following manufacturer's protocol (NEB) for 2.5 hrs. at room temperature. Two μL of ligation reaction was used to transform 30 μL of NovaBlue singles E. coli competent cells (Novagen) following manufacturers protocol. Randomly picked colonies were screened for the correct constructs by plasmid sequencing. Sequence specificity of each miR-shRNA within the cluster was altered to the indicated native miRNA sequences below by substituting the mature miRNA sequence with the mature native target miRNA sequence using a site-directed method approach as described. Two primers in opposite direction were designed so that the sequences that are to be altered were incorporated in a long forward primer where the loop and last 3'-20 nt sequences were the native miRNA sequence for primer binding. All secondary structures (bulges, wobble-pairs and mature miRNA length), loop and the 20 nt 3'-flank of the parental miRNA sequences were preserved. As for the reverse primer, it is specific to the 5'-region of the native miRNA sequence. The primer sets for each hairpin and the orders of the native and modified hairpins are shown below: Native miR-302 cluster hairpins in the order of their presence in the transcript and their modified variants are listed below. The native miR-302 cluster (302b, 302c, 302a, 302d, and 367) was modified by replacing each of the miRNA hairpin with different miRNA

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hairpins. For example, miR-302b hairpin was replaced with miR-26b which resulted in a 26b, 302c, 302a, 302d, and 367 cluster.

Forward-302b/26b (80 nt):

5’TTCAAGTAATTCAGGATAGGTTgtgactttaaaATCCTGTCCTGAATTACAAGGTAGgagtgaatccaa tttacttctcc-3’

ANTISENSE (GUIDE) STRAND: TTCAAGTAATTCAGGATAGGTT

Loop sequence: gtgactttaaa

SENSE (PASSENGER) STRAND: ATCCTGTCCTGAATTACAAGGTAG

Reverse-302b (23 nt) Phosphorylated: 5’-tgaagggagcccacccaacatac-3’

302 wt: (302b, 302c, 302a, 302d, and 367

302c/26b: (302b, 126, 302a, 302d, and 367)

Native miR-302c was altered to miR-126

Forward miR-302c/126 (77 nt):

5’CATTATTACTTTTGGTACGCGtgtgaaacaaaagCGTGCACCAAAAGTAATATCGGTGGaggtgtctc caagccagc-3’

ANTISENSE (GUIDE) STRAND: CATTATTACTTTTGGTACGCG

Loop sequence: tgtgaaacaaaag

SENSE (PASSENGER) STRAND: CGTGCACCAAAAGTAATATCGGTGG

Reverse -302c (24 nt) Phosphorylated: 5’-gcaaaggggatcccttcaaatgag

302 wt: (302b, 302c, 302a, 302d, and 367

302a/423: (302b, 302c, **423, 302d, and 367)**

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Native miR-302a was altered to miR-423

Forward-miR-302a/423 (77 nt):

5'AGCTCGGTCTGAGGCCCCTCAGgaaactaaaTTGAGGGGCCTGAGACTGAGCAggtgatggtaagtctt cctttttac-3'

ANTISENSE (GUIDE) STRAND: AGCTCGGTCTGAGGCCCCTCAG

Loop sequence: gaaactaaa

SENSE (PASSENGER) STRAND: TTGAGGGGCCTGAGACTGAGCA

Reverse-302a (23 nt) Phosphorylated: 5'-agtggtggggagcccagtcgttg

Cell culture and lentiviral vector transduction. HEK 293T cells were purchased from the American Type Culture Collection (ATCC, Manassas, VA) and maintained in high-glucose (4.5 g/liter) Dulbecco's modified Eagle's medium (DMEM) supplemented with 2mM glutamine and 10% fetal bovine serum (FBS). Lentiviral transgene vectors with appropriate miRNA inserts (either as single or multiple miR-302 hairpins) were packaged in HEK 293T cells by co-transfecting cells with various lentivirus packaging plasmids [70]Sun, 2006 #1722} by calcium phosphate precipitation as previously described [20,71]. The viral supernatants were collected 48 hrs. after transfection, concentrated via ultracentrifugation, and stored at -80°C until use. Virus titers were determined by transduction of HEK293T cells with serially diluted viral partciles and monitoring of percentage of red fluorescent protein (RFP) or green fluorescent protein (GFP) positive cells by microscopy.

Real time quantitative RT-PCR to assess lentiviral miR-302 expression in transduced cells. HEK293T cells were seeded in 96-well format with 5,000 cells / well cultured for 24 h, then spin infected (3000 rpm for 20 min) with 8 μg/mL polybrene with lenti-miR-302 viral particles at an MOI of 1. Total RNA was isolated 72 hrs. post-infection using miRvana RNA Isolation kit (Ambion) following manufacturer's instructions. The total RNA was then resuspended in nuclease-free water. Residual DNA was digested with Ambion TURBO DNase (Life Technologies, Carlsbad, CA) with 1 μg of total RNA in a 10 μL reaction, in accordance with the manufacturer's instructions. miRNA expression was analyzed by qRT-PCR in triplicate using microRNA detection system (Applied BioSystems, Biosystems, Foster City, CA) with 100 ng of DNase treated total RNA and a reverse transcription (RT) primer pool was created with specific miRNA RT primers and qRT-PCR was performed according to the manufacturer's instructions. Real-time PCR was

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carried out with 3 µL of RT reaction. Three µL of RT reaction was added into each well containing 6 µL of the 1:100 diluted TaqMan® MicroRNA Assays 5xRT primer pool and RT reaction mix for a total reaction volume of 15 µL using TaqMan® MicroRNA RT kit (Life Technologies, 4366596) and incubated as indicated by the manufacturer's protocol. The cDNA was then pre-amplified through 12 cycles using 3.75 µL of 1:100 diluted pool of primers (20x TaqMan® MicroRNA Assays), 2.5 µL RT Product and 12.5 µL TaqMan® PreAmp Master Mix (2X) in a final volume of 25 µL following manufacturer's protocol (Life Tech., 4391128) and amplified per manufacturer's instructions. For real-time PCR reactions, 0.1 µL of the 1:8 diluted preamplification products were amplified at a final volume of 10 µL by qRT-PCR with 5 µL of 2x TaqMan® Universal Master Mix II (Life Tech., 4440048), 0.5 µL of 20 x TaqMan® MicroRNA Assay following manufacturer's instructions using an ABI PRISM 7000 Fluorescent Quantitative PCR System (Apllied Biosystems, Foster City, CA) according to the standard procedures. The relative miRNA expression was calculated using the $2^{-\Delta Ct}$ method. U6 snoRNA was used for sample-to-sample normalization and data was presented as relative intensity using the comparative Ct method. Quantitative RT-PCR primer sequences for U6 RNA were as follows: U6: Forward: 5'-GCT CGC TTC GGC AGC ACA TAT ACT AA-3'; Reverse: 5'-ACG AAT TTG CGT GTC ATC CTT GCG-3'.

Statistical analyses. Statistical analysis was performed using Student's *t*-test for two sample comparisons and $P \leq 0.05$ was considered statistically significant. All statistical tests were conducted using Microsoft Excel® provided by Microsoft Corporation (Redmond, WA).

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COMPETING FINANCIAL INTERESTS

The author declare no competing financial interests.

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FIGURE LEGENDS

Table 1. Examples of human intronic single and polycistronic miRNA clusters, their features, and their functional results upon expressing them in HeLa cells. Intronic miRNAs were identified in several databases (http://microrna.sanger.ac.uk/; www.genome.ucsc.edu; http://miRnamap.mbc.nctu.edu.tw) and in literature [48,72] and cloned into the GFP coding sequence of the pGFP-miR-shRNA expression mammalian expression vector. Each construct was tested for their ability to induce correct mRNA splicing and subsequent translation of functional GFP and RNAi-induced silencing of *R-Luc*-tagged target gene expression. Relative *R-Luc* activity of HeLa cells tarsnfected with miR-GFP singleton or multimiR polycistrons or siRNAs specific to *R-Luc* as control were evaluated 48 hrs. after the transfection. Luciferase activity was normalized to *f-Luc* and and all referenced to cells transfected only with the reporter construct (pGI-GFP wt), which is set at 1.0. The data are presented as % knockdown ± Standard deviation (SD) of *R-Luc* activity. Average knockdown and SD represent three independent transfections. Statistical analysis was performed using Student's *t*-test for two sample comparisons and $P \leq 0.05$ was considered statistically significant. All statistical tests were conducted using Microsoft Excel® provided by Microsoft Corporation (Redmond, WA).

Luc. Luciferase. KD: Knockdown.

Figure 1. Strategy for monitoring intronic miR-shRNA-induced gene silencing and functional GFP expression in mammalian cells. Architecture of intronic miR-shRNA-transgene and *R-Luc*-tagged reporter transcripts used to induce RNAi and to measure the RNAi activity in HeLa cells are

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shown. Illustration of the miR-shRNA-GFP (**Top left**) and the dual-luciferase miR-shRNA reporter vectors (psiCHECK^{TM}-2), (**Top right**) are shown. The miR-shRNA-GFP vectors provide a quantitative and rapid approach for examining the expression and subsequent RNAi activity as well as expression of functional protein coding sequence (*e.g.* GFP) in mammalian cells. The psiCHECK™ -2 vectors provide a quantitative and rapid approach for examining the effect of 3′ untranslated regions (3′ UTRs), such as miRNA target sequences, on gene expression. psiCHECK™ vectors contain a multiple cloning region downstream of the stop codon of an SV40 promoter-driven Renilla luciferase (*R-Luc*) gene. This allows expression of a *R-Luc* transcript with the 3′ UTR sequence of interest. *R-Luc* activity is then used as a measure of the effect of the 3′ UTR on transcript stability and translation efficiency. The schematic illustrates the processing of the miR-shRNA-GFP RNA transcript leading to functional mRNA splicing and generation of functional miRNA/siRNA (**Lower left**) and *R-Luc*-tagged reporter transcript (**Lower right**) in the same mammalian cells co-transfected with these vectors. *R-Luc*-tagged reporter vectors contain a multiple cloning region downstream of the stop codon of an SV40 promoter-driven *R-Luc* gene. By appending the miR-shRNA target sequence of interest to the *R-Luc* gene, luciferase activity can be used as a marker for measuring the activity of miR-shRNAs expressed in the same cell from the miR-shRNA-GFP expression vector. Because miRNA targets are designed to have full complementarity to their cognate miRNAs, it is expected that RNAi induced silencing of *R-Luc* expression occurs via the cleavage and subsequent degradation of the *R-Luc* RNA. Thus, measuring decreases in *R-Luc* activity provides a quantitative way of monitoring RNAi effects in cells. The second reporter gene built in the Luc reporter vector, firefly luciferase (*f-Luc*), is used to

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normalize *R-Luc* expression and its expression is not affected by RNAi activity. (A)N: Simian virus 40 polyadenylation signal. *R-Luc*: Renilla luciferase. *f-Luc*: firefly luciferase. Pre-miRNA: precursor miRNA.

Figure 2. Schematic of construction intronic single or multi-miR-shRNA and transgene vectors. The workflow shows the architecture and cloning strategy of the intronic single or multiple miRNA cassettes into p-GI-GFP vector. pri-miRNA sequences were PCR amplified using primers (**Supplementary Table 1.**) that were complementary to the regions outside of the intron splice donor (SD) and splice acceptor (SA) junctions and cloned into unique SacI/SacII and BstEII site of the GFP transgene in place of the GI intro. Arrows depict the relative position of the primers relative to the splice junction of the natural introns of each pri-miRNA sequence. The structure of intronic miRNAs were depicted as transcripts of single or multiple miRNAs. Vectors were created with one promoter option; CMV (cytomegalovirus). SD and SA denote splice donor and splice acceptor sequences of the introns. Restriction enzymes Sac I and Sac II, and Bste II are unique to the host vector and the intronic miRNA sequences.

Figure 3. The simultaneous delivery of intronic miR-423 and GFP gene produces functional miRNAs and GFP protein in HeLa cells. (**A**) Illustration of single miR-423-GFP and multi-miR-302 cluster where miR-302a hairpin was replaced with miR-423 by site directed mutagenesis. (**B**) Knockdown of *R-Luc* activity in reporters specific to miR-423. Cells co-transfected with pGI-423-GFP and the psiCHECK-2 reporter containing sites complementary to miR-423 show a significant decrease in *R-Luc* activity compared to cells co-transfected with the pGI-GFP wt (no miRNA sequence). *R-Luc* and *f-Luc* luciferase activity were measured 48 hrs. after transfection and the

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ratio of *R-Luc* to *f-Luc* were all referenced to cells transfected only with the GFP reporter construct (pGI-GFP wt) which lacks the miR-shRNA sequence, which is set at 1.0. *P* values for each miRNA or siRNA that showed specific and potent silencing of *R-Luc*-tagged target activity are shown. Each experiment was replicated at least three times. Each bar represents mean (± standard deviation, SD) for each group. Note that error bars are very tight and may not be visible. (**C**) Detection of GFP fusion protein with fluorescence microscopy in HeLa cells transfected with plasmids encoding either the multi-miR-302 cluster cassette engineered to express miR-423 and GFP or single miR-423-GFP plasmid (pGI-miR-423-GFP). Cells were transfected with respective plasmids and imaged using fluorescence microscopy 24 hrs. after transfection. Solid black bars represent miRNA or siRNA specific suppression of *R-Luc* activity. The *P* values of the control vector expressed miRNAs either in a cluster or as singletons and siRNAs targeting *R-Luc* are: $P \leq 0.001$.

Figure 4. Natural intronic-single miRNAs co-expressed with reporter GFP gene on a single transcript exert robust and specific RNAi mediated silencing of *R-Luc*-tagged target sequences. HeLa cells were transfected with intronic-miR-shRNA expression plasmids and the luciferase reporter plasmids. The *R-Luc* and *f-Luc* luciferase activity were measured 48 hrs. after transfection and the ratio of *R-Luc* to *f-Luc* were normalized to pGI-GFP empty vector which lacks the miR-shRNA sequence, which is set at 1.0. *P* values for each miRNA or siRNA that showed specific and potent silencing of *R-Luc*-tagged target activity are shown. siRNA targeting *R-Luc*iferase was used at 100 nM concentration as a control. Two sequence confirmed clones were

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used for functional testing of each target specific intronic-miRNA. Averages and standard deviation (SD) represent three independent transfections. Error bars represent the SD of three independent transfections. Note that error bars are very tight and may not be visible. Solid black bars represent miRNA or siRNA specific suppression of *R-Luc* activity. Representative *P* values calculated using the t-test for two sample comparisons are shown. The *P* values of the vector expressed miRNAs either in a cluster or as singletons and siRNAs targeting *R-Luc* are noted in the figure.

Figure 5. Intronic miRNA 106 polycistron co-expressed with reporter GFP gene on a single transcript promote robust and specific RNAi mediated silencing of *R-Luc*-tagged target sequences. HeLa cells were transfected with intronic-miR-106-cluster expression plasmids and the luciferase reporter plasmids and the *R-Luc* and *f-Luc* luciferase activity were measured 48 hrs. after transfection and the ratio of *R-Luc* to *f-Luc* were normalized to pGI-GFP empty vector which lacks the miR-shRNA sequence. siRNA targeting *R-Luc*iferase was used at 100 nM concentration as a control. Two sequences confirmed clones were used for functional testing of each target specific intronic-miRNA. Averages and standard deviation (SD) represent three independent transfections. Error bars represent the SD of three independent transfections. Note that error bars are very tight and may not be visible. Solid black bars represent miRNA or siRNA specific suppression of *R-Luc* activity. Solid black bars represent miRNA or siRNA specific suppression of *R-Luc* activity. Representative *P* values calculated using the t-test for two sample comparisons are shown. The *P* values of the vector expressed miRNAs either in a cluster or as singletons and siRNAs targeting *R-Luc* are noted in the figure.

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Figure 6. Intronic miRNA-302 polycistron co-expressed with reporter GFP gene on a single transcript induce robust and specific RNAi mediated silencing of *R-Luc*-tagged target sequences. HeLa cells were transfected with intronic-miR-302-cluster expression plasmids and the luciferase reporter plasmids and the *R-Luc* and *f-Luc* activity were measured 48 hrs. after transfection and the ratio of *R-Luc* to *f-Luc* were to normalized to pGI-GFP empty vector which lacks the miR-shRNA sequence as described in Materials and Methods. siRNA targeting *R-Luc*iferase was used at 100 nM concentration as a control. Two sequences confirmed clones were used for functional testing of each target specific intronic-miRNA. Averages and standard deviation (SD) represent three independent transfections. Error bars represent the SD of three independent transfections. Note that error bars are very tight and may not be visible. Solid black bars represent miRNA or siRNA specific suppression of *R-Luc* activity. Solid black bars represent miRNA or siRNA specific suppression of *R-Luc* activity. Representative *P* values were calculated using the t-test for two sample comparisons are shown. The *P* values of the vector expressed miRNAs either in a cluster or as singletons and siRNAs targeting *R-Luc* are noted in the figure.

Figure 7. Individual mature miRNAs expressed from the lentiviral vectors expressing miR-302 polycistron are detectable by qRT-PCR. HEK293T cells were seeded in 96-well format with 5,000 cells / well cultured for 24 h, then spin infected (3000 rpm for 20 min) with 8 μg/mL polybrene with lenti-miR-302 viral particles at an MOI=1. Total RNA was isolated 72 hrs. post-infection using miRvana RNA Isolation kit (Ambion) and analyzed by qRT PCR using miRNA specific kits from Applied BioSystems. Data show that cells transduced with viral particles express the intended miRNA, with RFP positive cells ranging from 20-50% of the culture. Note that

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HEK293T cells do not express miR-302; hence, all miRNA expression is due to lenti-viral vector. Middle and right panel: brightfield and fluorescent imaging of cells expressing Lenti-RFP-miR-302 vector. Averages and standard deviation (SD) represent three independent transfections. Error bars represent the SD of three independent transfections. Note that error bars are very tight and may not be visible. Solid black bars represent miRNA or siRNA specific suppression of *R-Luc* activity.

Figure 8. Substitution of intronic miR-302c with miRNA-423 effectively silence its *R-Luc*-tagged target sequence. (**A**) Individual native miRNA hairpins within the intronic miR-302 cluster (miR-302b/302c/302a/302d/367) were replaced with individual miRNA hairpins such as miR-423, miR-26b, miR-126, and miR-7-1. These single intronic miRNAs were found to exert robust RNAi activity when tested individually. The engineered miRNA expression cassettes including **miR-302 wt:** 302b/302c/302a/302d/367; **miR-302a/423:** 302ab/302c/**423**/302d/367; **miR-302b/26:** **26**/302c/302a/302d/367; **miR-302c/126:** 302a/**126**/302a/302d/367; and **miR-302d/7-1:** 302a/302c/302a/**7-1**/367 were tested for their ability to silence their cognate targets using *R-Luc* reporter system. HeLa cells were transfected with individual intronic-miRNA cluster expression plasmids and the luciferase reporter plasmids and the *R-Luc* and *f-Luc* luciferase activity were measured 48 hrs. after transfection and the ratio of *R-Luc* to *f-Luc* were normalized to pGI-GFP empty vector which lacks the miR-shRNA sequence. Data show that miR-423 specifically silenced *R-Luc*-tagged target expression in the same cell comparable to pGI-423 control which expresses natural single pri-miR-423 imbedded in the GI intron. Averages and standard deviation (SD) represent three independent transfections. Error bars represent the SD of three independent transfections. Note that error bars are very tight and may not be visible. Solid black bars represent

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miRNA or siRNA specific suppression of *R-Luc* activity. Not all pre-miRNA sequences within the miR-302 polycistron are amenable for replacement with another pre-miR-shRNA. Only the anti-423 miR-shRNA inhibits *R-Luc* reporter comparable to pGI-423 positive control plasmid which expresses natural pri-miR-423 from an artificial intron. miR-302b, 302c, and 302d which were replaced with miR-26, 126, and 7-1 pre-miRNA where native duplex and loops sequences were retained did not show inhibitory activity. (**B**) The original miR-shRNAs within the miR-302 polycistron maintains their RNAi activity and functional GFP expression after replacing the indicated miR-shRNAs with another miR-shRNA hairpin and GFP expression. Only the anti-423 miR-shRNA inhibits *R-Luc* reporter comparable to pGI-423 positive control plasmid which expresses natural pri-miR-423 from an artificial intron. miR-302a, 302c, and 302d which were replaced with miR-26, 126, and 7-1 pre-miRNA where native duplex and loops sequences did not show inhibitory activity. However, all the original miR-shRNAs maintained their activity and none of them silenced non-cognate targets. siRNA targeting *R-Luc* was used at 100 nM concentration as a control. *R-Luc* expression was measured 48 hrs. after transfection and normalized to pGI-GFP empty vector which lacks the miR-shRNA sequence. Error bars represent the standard deviation (SD) of three independent experiments. Representative *P* values calculated using the t-test for two sample comparisons are shown. The *P* values of the vector expressed miRNAs either in a cluster or as singletons and siRNAs targeting *R-Luc* are noted in the figure.

Figure 9. Number of miRNAs present in miR-302 cluster affect viral titer. As illustrated in the figure, the results from this study demonstrated that more miRNA is not always better when packaging multi-miRNA transcripts from a lentiviral vector. Lentiviral vectors expressing

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anywhere from one (miR-302a) to five (miR-302a,-302b-3-2c-302d and 367) miR-302 hairpins were used to transfect HEK293T cells and viral particles were collected 48 hrs. after transfection and viral titer was determined on HEK293T cells 2-5 days after infection as described in Materials and Methods. Lentiviral transgene expressing five hairpin cluster (miR-302a,-302b-3-2c-302d, and 367) resulted in $6x10^4$ packaged viral particles (transduction unit (TU)/mL) whereas transgene containing four ($1.75x10^6$ TU/mL), three ($2.4x10^6$ TU/mL), two ($4.25x10^6$ TU/mL), and one ($5.5x10^6$ TU/mL) resulted in the fewest number of packaged viral particles. There was a linear inverse correlation between viral titer and the number of hairpins present in the transgene (R^2 = 0.986). TU: Transduction unit.

Figure 10. Inducible knockdown of Drosha interferes with miRNA microprocessing but not with exon splicing and subsequent expression of the reporter gene (*e.g.* RFP: Red Fluorescent Protein). A stable Drosha knockdown 293T cell line was generated that express Drosha specific shRNAs [infected at multiplicity of infection (MOI) of 1, selected with puromycin (1 mg/mL) for 6 days and induced by doxycycline (Dox, 1 mg/mL) for 48 or 96 h]. The expression of mature miRNA was quantified by qRT-PCR at 48 and 96 hrs. after Dox induction. As shown above, Ct values of mature miRNA increases as Drosha knockdown is induced. Ct value is inversely correlated with mature miRNA levels assessed by qRT-PCR. Data show that upon induction of Drosha knockdown, miRNA expression was reduced by at least 3.5 fold at 48 and 96 hrs. after the beginning of induction of shRNA expression. RFP: Red Fluorescent Protein. TU: Transduction unit. MOI: Multiplicity of infection. Dox: Doxycycline. qRT-PCR: Quantitative reverse transcription polymerase chain reaction. Ct: threshold cycle.

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Table 1. Seyhan A

Intronic-miRNAs	GFP expression	% Knockdown (average of three)	Average ± SD	*P* values
miR-26b	No	88	0.008	8.89×10^{-3}
miR-126'5	Yes	92	0.002	1.10×10^{-7}
miR-126'3	Yes	97	0.003	2.28×10^{-4}
miR-330	Yes	71	0.014	5.97×10^{-2}
miR-423	Yes	85	0.007	5.62×10^{-4}
miR-7-1	Yes	92	0.002	8.15×10^{-3}
miR-208a	No	77	0.002	2.76×10^{-5}
Intronic-miRNA clusters				
miR-302a-5'	Yes	94	0.002	3.1×10^{-5}
miR-302a-'3	Yes	80	0.004	8.9×10^{-6}
miR-302b-5'	Yes	52	0.040	6.7×10^{-3}
miR-302b-3'	Yes	92	0.040	7.8×10^{-3}
miR-302c-5'	Yes	0	0.013	1.8×10^{-3}
miR-302c-3'	Yes	70	0.006	2.78×10^{-6}
miR-302d	Yes	94	0.003	1.46×10^{-8}

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miR-367	Yes	97	0.001	$2.58x10^{-8}$
miR106b	Yes	48	0.002	$2.45x10^{-4}$
miR-93	Yes	69	0.009	$5.37x10^{-4}$
miR-25	Yes	67	0.002	$3.83x10^{-5}$

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Figure 1. Seyhan A

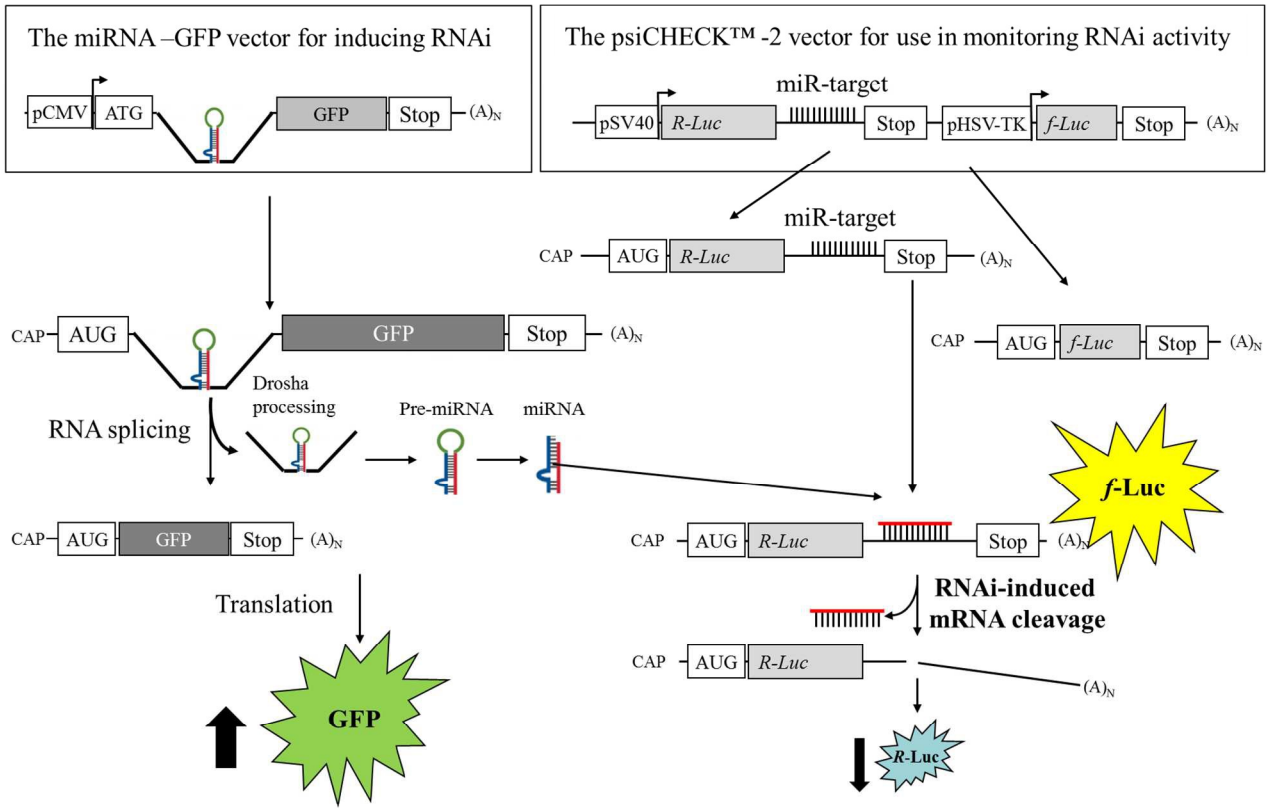

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Figure 2. Seyhan A

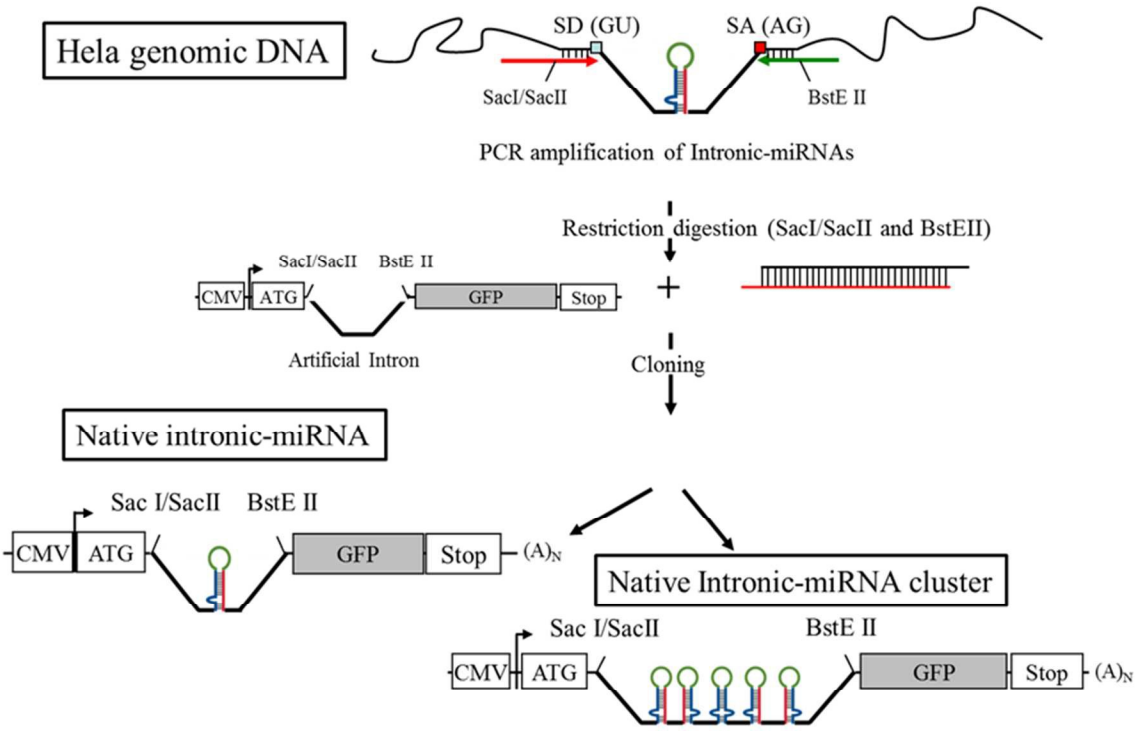

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Figure 3. Seyhan A

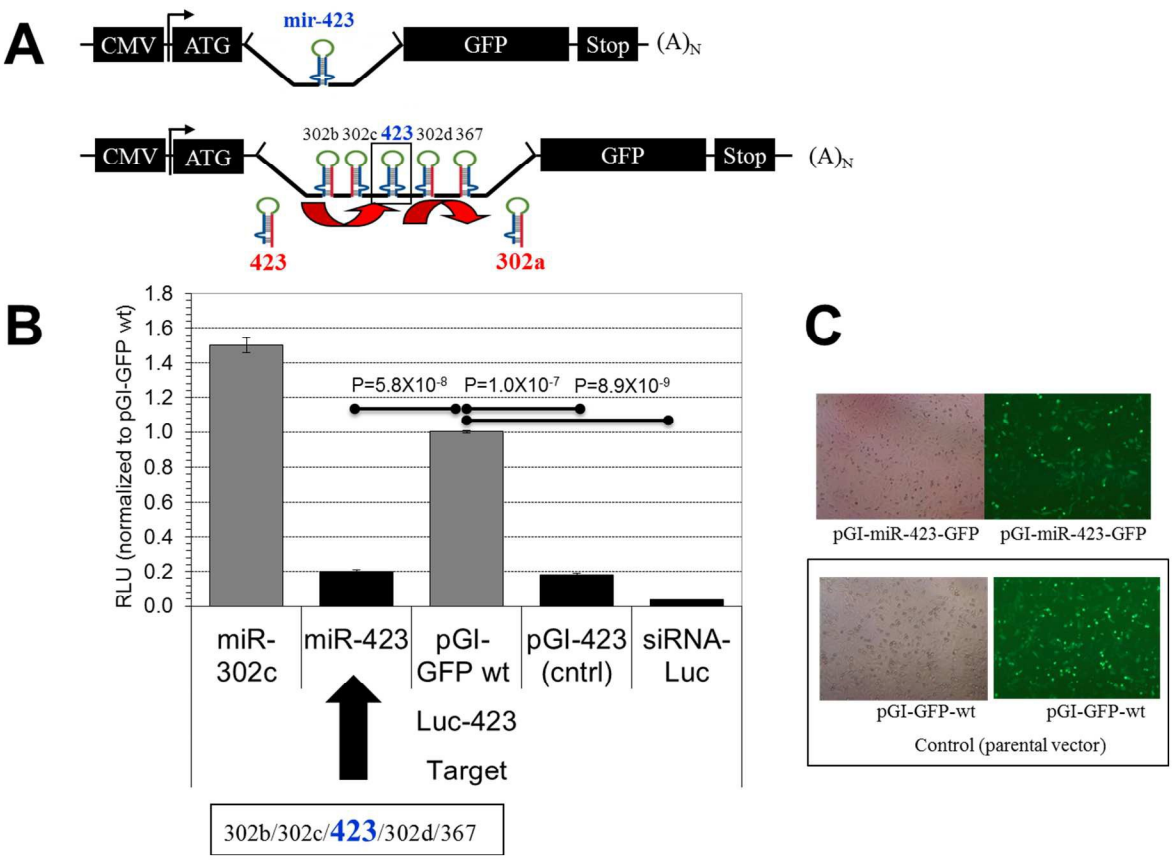

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Figure 4. Seyhan A

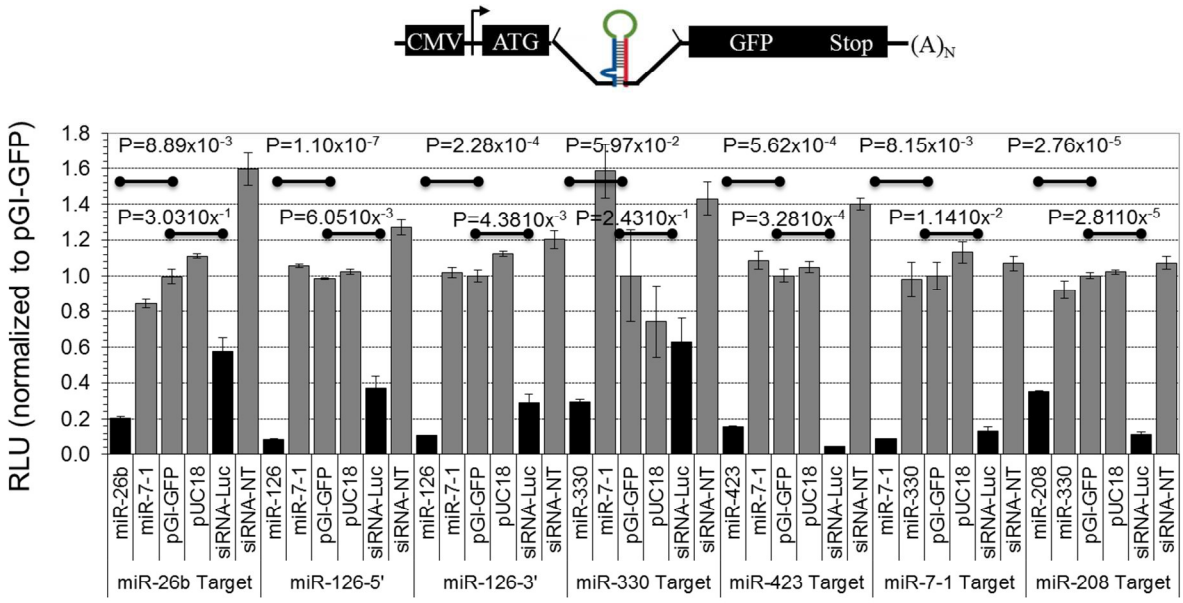

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Figure 5. Seyhan A

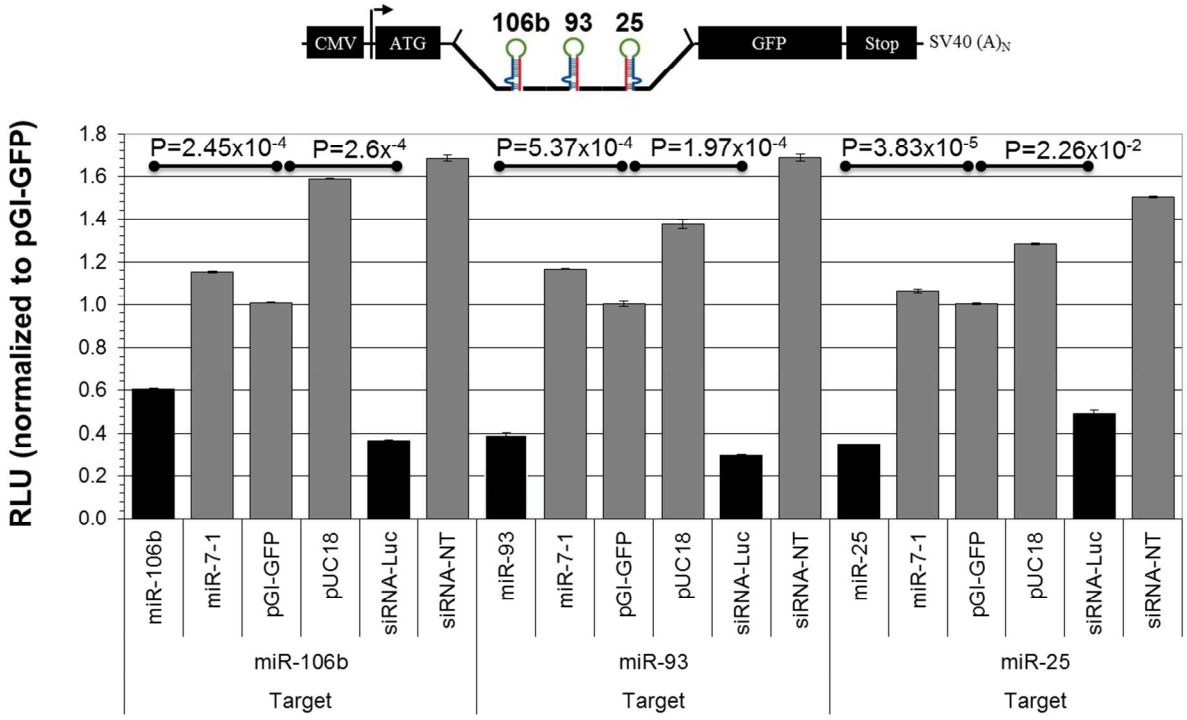

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Figure 6. Seyhan A

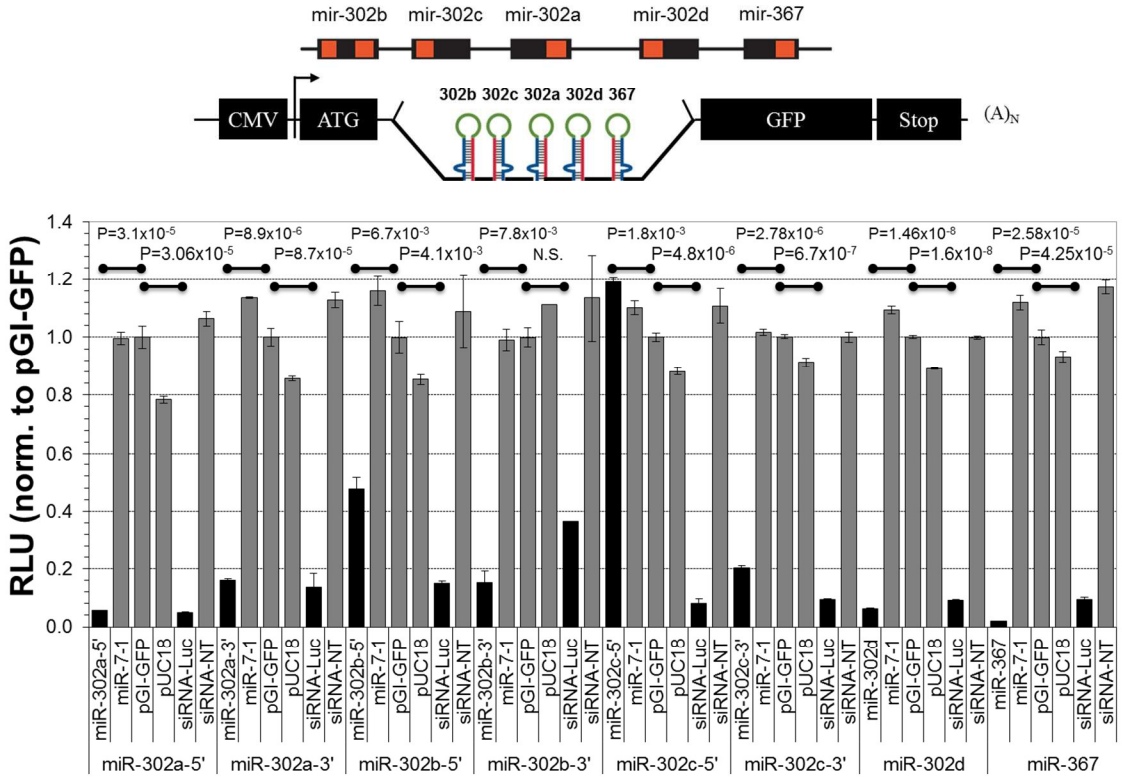

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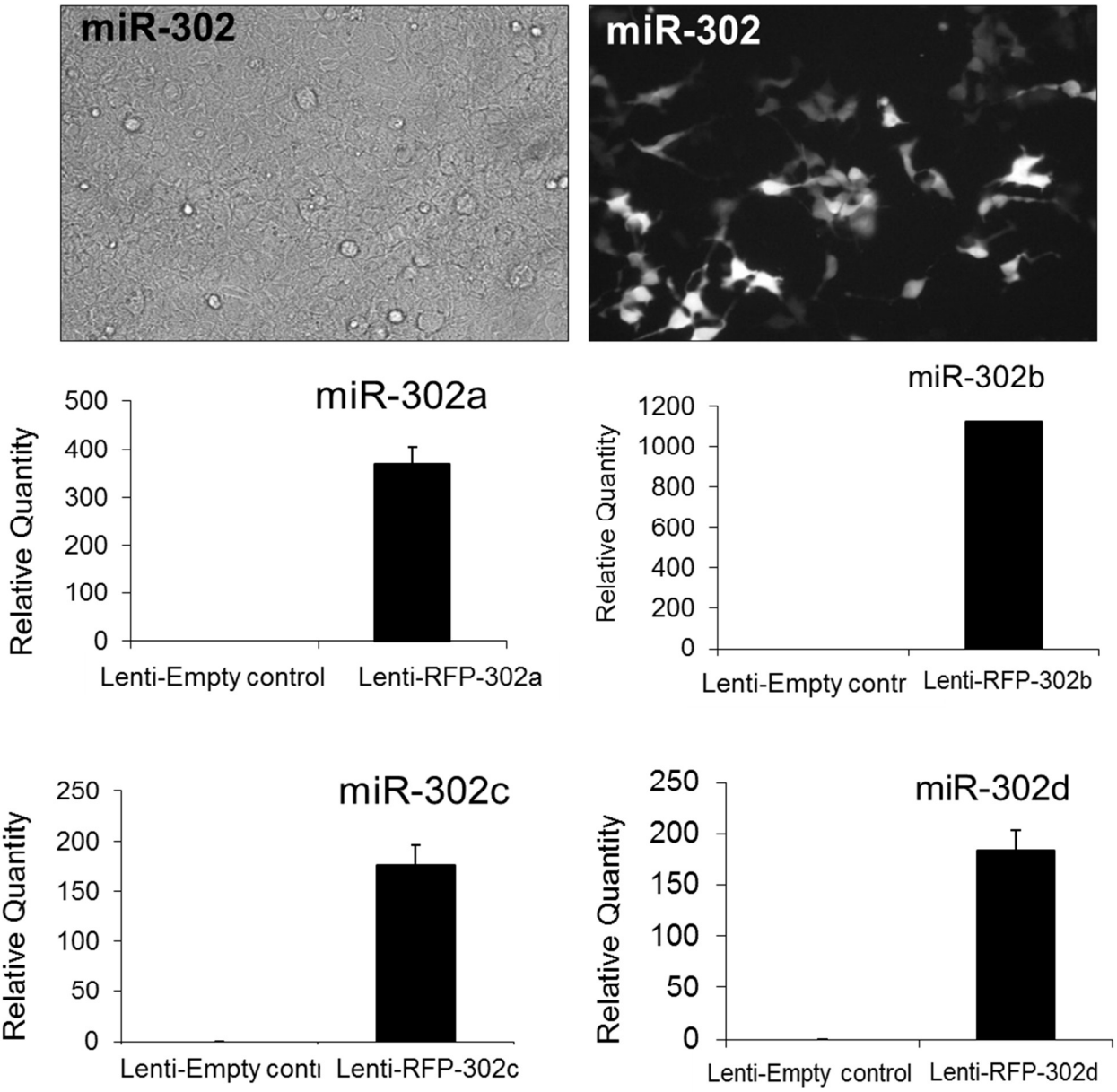
Figure 7. Seyhan A



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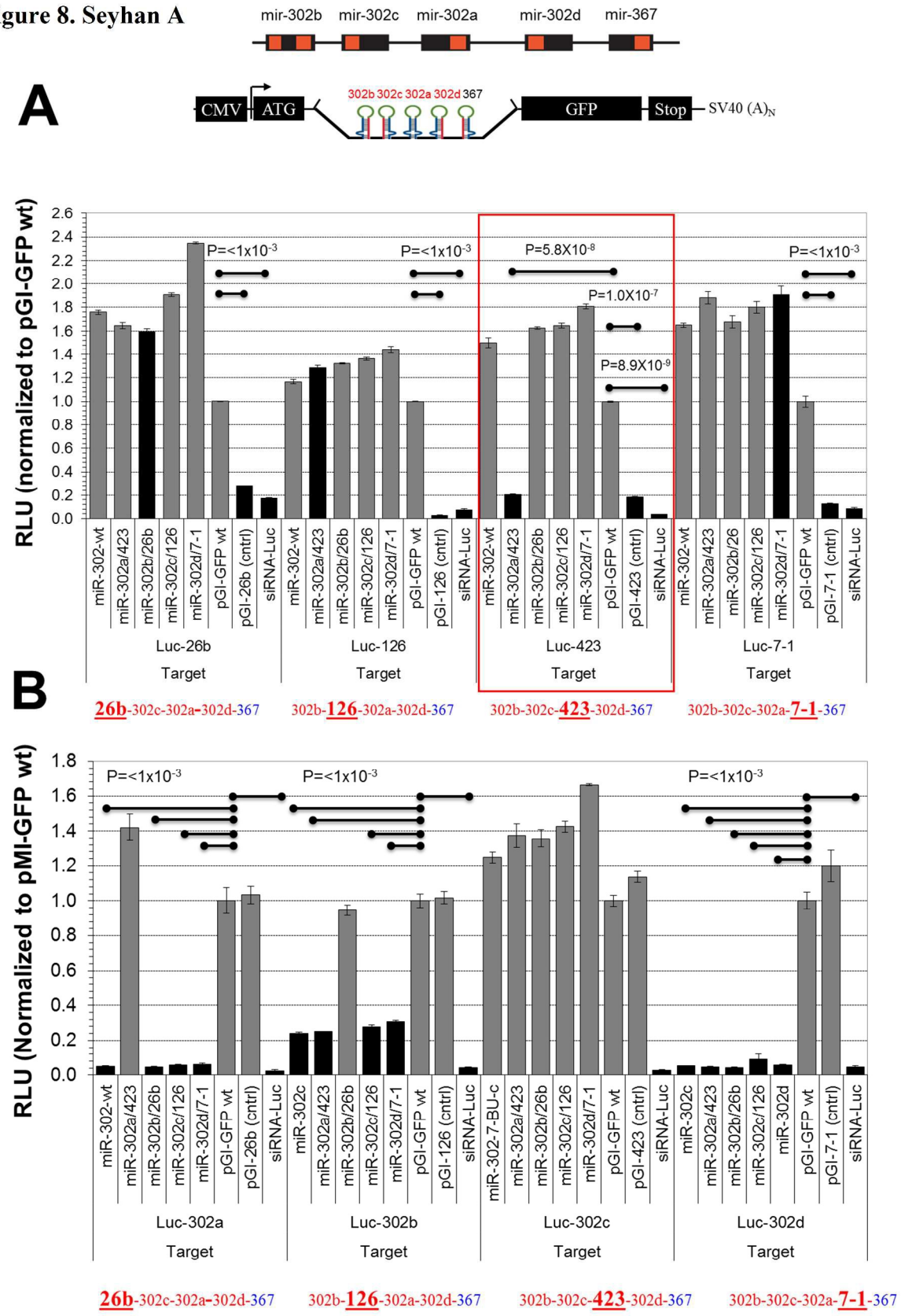

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Figure 9. Seyhan A

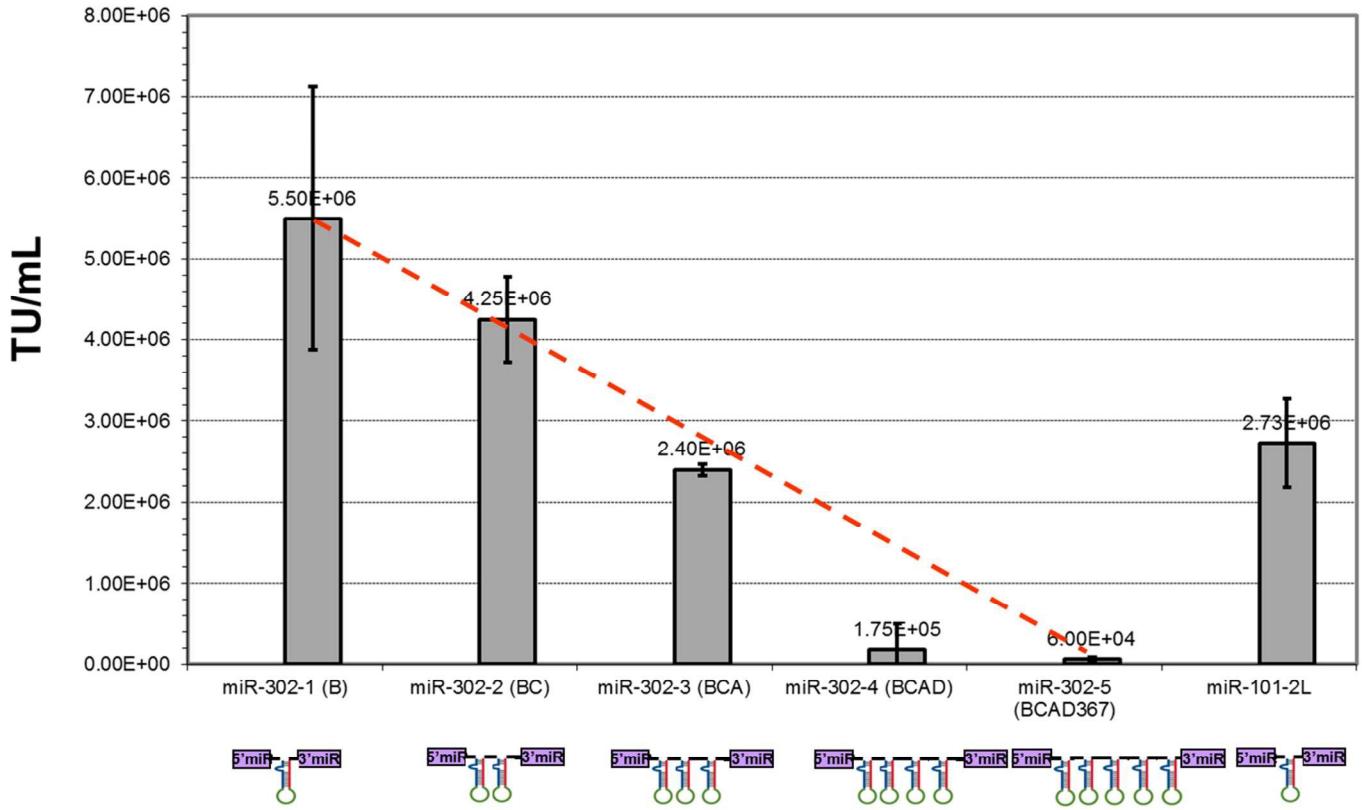

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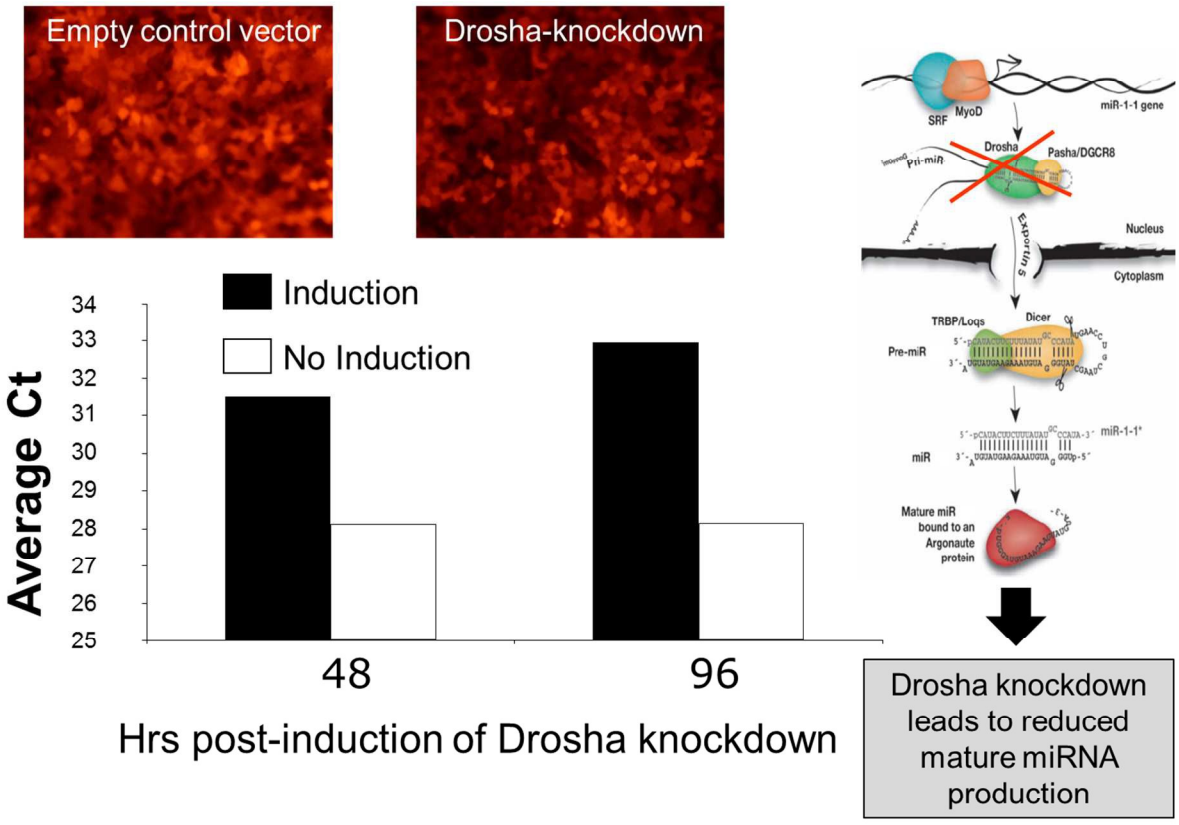
Figure 10. Seyhan A



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