

High-efficiency Generation of Multiple Short Noncoding RNA in B-cells and B-cell-derived Extracellular Vesicles

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Short noncoding (snc)RNAs are important new players in the landscape of biologics with therapeutic potential. Recently, we reported on a new method for the synthesis and delivery of snc RNA in B-cells transfected with plasmid DNA. Here using the same approach, we demonstrate that B-cells can be programmed for the enforced biogenesis and synchronous release of multiple sncRNAs. Our data show that this goal is feasible and that multiple sncRNA are released in the extracellular compartment in amounts comparable to those from B-cells programmed to express and secrete one sncRNA only. Furthermore, we found that the cargo of extracellular vesicles (EVs) isolated from programmed B-cells is remarkably enriched for multiple sncRNA. On average, we found that the content of multiple sncRNAs in EVs is 3.6 copynumber/EV. Collectively, we demonstrate that B-cells can be easily programmed toward the synthesis and release of multiple sncRNAs, including sncRNA-laden EVs, efficiently and specifically.

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Introduction

In eukaryotes, evolutionarily conserved 20–30 nucleotides noncoding RNAs (miRNAs) regulate gene expression by binding to sequences with partial complementarity at the 3'-UTR of target RNA transcripts, causing translational repression and/or messenger RNA degradation.^{1,2} Each miRNA may repress up to hundreds of transcripts hence regulating a large portion of the transcriptome.³ miRNAs are involved in the regulation of a variety of processes including cell growth, metabolism, immunity, inflammation, and cancer.^{4–7}

The explosive pace at which the field has developed in the past decade and the vast potential for medical intervention have spurred efforts in developing delivery systems that could be translated to the clinic. Although *in vivo* delivery of free or encapsulated synthetic oligonucleotides has been demonstrated in many instances,^{8–11} miRNA-based interventions based on systemic delivery are still hampered by hurdles such as rapid uptake by scavenger macrophages, degradation by serum and tissue nucleases, as well as safety and cost considerations.

Recently, this laboratory developed a new system for the synthesis and delivery of short, noncoding RNAs for therapeutic purposes. The new approach consists in the use of autologous primary B-lymphocytes that can be programmed by transfection with suitably engineered plasmid DNA to the biogenesis and release of sort noncoding (snc)RNA molecules.¹² We reported that sncRNAs are secreted in 24 hours, both as free molecules and cargo in extracellular vesicles (EVs). EVs were further shown to undergo *in vitro* and *in vivo* internalization by third party cells, causing marked (~70%) target downregulation.

Reasoning that in many clinical situations a multi-pronged sncRNA approach would be desirable, here we tested the possibility of programming B-cells simultaneously for biogenesis and secretion of multiple sncRNAs, including their release as EV cargo. In recent years, only few reports demonstrated the expression of multiple sncRNAs in cells using either a retrovirus or plasmid DNA,^{13–15} but no attempts were made to assess the release of the sncRNAs in the extracellular compartment or their inclusion in EVs. The results show that B-cells transfected with plasmid DNA carrying the nucleotide sequence of multiple sncRNAs in tandem undergo the simultaneous biogenesis and secretion of multiple sncRNA, including their release and incorporation in EVs, at high efficiency and specifically.

Results

Engineering plasmids comprising nucleotide sequences of multiple sncRNAs

We previously showed that primary murine B-lymphocytes and model murine B-cells transfected with plasmid DNA pCMV/mir carrying the nucleotide sequence of anti-miR-150, are reproducibly programmed for the synthesis and secretion of anti-miR-150.¹² Here we verified that B-cells can be programmed for the synthesis and secretion of multiple sncRNAs simultaneously. To this end, we prepared a panel of five DNA plasmids each comprising either one or two sncRNA nucleotide sequences for their precursor miR (pre-miR) stem loop. As a model system, we used miR-150, miR-155, and anti-miR-155, which are relevant to the regulation of T-cell memory.¹⁶ Specifically, we generated two plasmids, one carrying in tandem miR-150 and miR-155; the other carrying in tandem miR-150 and anti-miR-155. Plasmids carrying miR-150, miR-155, and anti-miR-155 alone served as reference.

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The precursor stem loop for each pre-miR sncRNA and final individual plasmids bearing precursor sncRNAs as single or tandem elements are illustrated in **Figure 1**.

Synchronous intracellular expression of two short noncoding RNA

We probed intracellular sncRNA expression in murine J558L myeloma cells transfected by Amaxa electroporation and cultured for 48 hours after transfection. Total RNA was extracted and tested by RT-qPCR as described in Materials and Methods. The expression of miR-150 and miR-155 in J558 cells transfected with pCMVmir carrying the two sncRNAs in tandem (combo) was comparable to that of J558L cells transfected with pCMVmir carrying only one of the corresponding sncRNAs (**Figure 2a**). Similarly, the expression of miR-150 and anti-miR-155 in J558L cells transfected with pCMVmir carrying these two sncRNA in tandem was comparable to that of J558L cells transfected with pCMVmir carrying only

one of the corresponding sncRNAs (**Figure 2b**). These results show, therefore, no obvious loss of efficiency in the biogenesis and expression of sncRNA in B cells programmed using a plasmid carrying nucleotide sequences for multiple sncRNAs.

Synchronous release of multiple sncRNAs in the extracellular compartment

A distinctive feature of our system is that B-cells transfected with a single sncRNA-carrying plasmid are very efficient at releasing sncRNA in the extracellular compartment at levels that, on a per molecule basis, are markedly higher than in the intracellular compartment. Since a 7AAD analysis of J558L cells day 2 or 5 after transfection failed to show any appreciable increase of cell death relative to control cells, the results support an active export mechanism.¹² Here we verified whether this principle would also apply to B-cells programmed for tandem sncRNA expression. The levels of miR-150 and

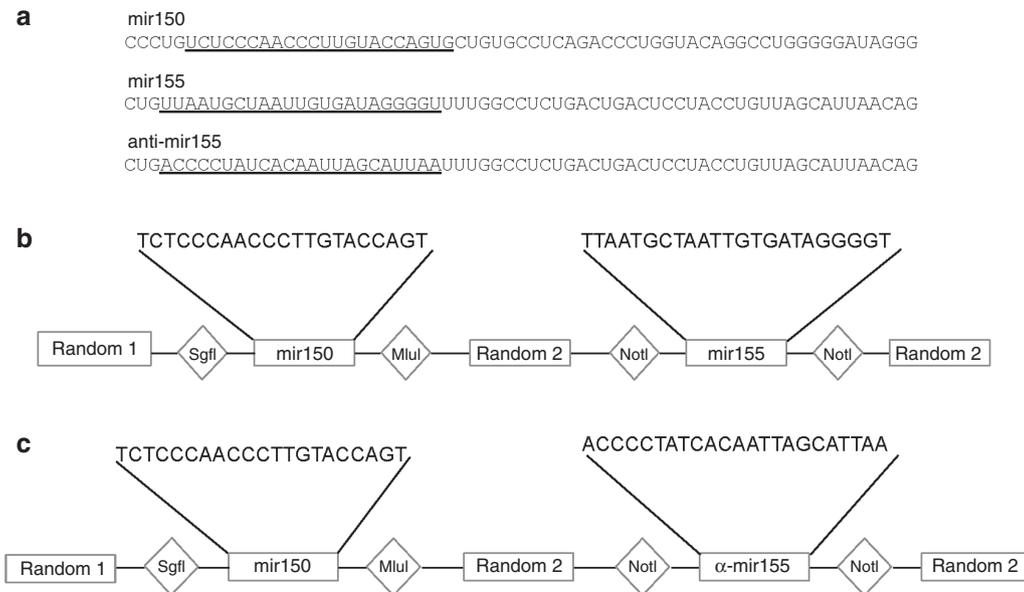


Figure 1 Schematic representation of plasmids used in the study. (a) The pri-mir nucleotide sequence of miR-150, miR-155, and anti-miR-155; (b) Schematic view of pCMV miR-150—miR-155. (c) Schematic view of pCMV mir-150—anti-miR-155.

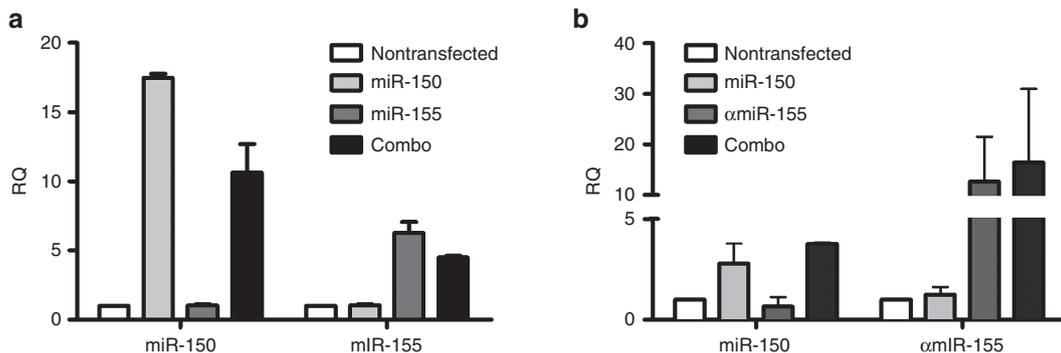


Figure 2 Expression of mir-150 and anti-miR-155 in programmed J558L cells. J558L cells were transfected with plasmid DNA encompassing the coding sequence for miR-150 alone, anti-miR-155 alone, or the combination of both (combo). Cells were harvested 36 hours after transfection and total RNA was extracted by the Zygem kit. cDNA was generated using RT-specific primers using the Lifetech microRNA assay kit. Samples were preamplified and then subject to quantitative reverse transcription polymerase chain reaction amplification using RT-specific primers. Results refer to the mean ± SD of two independent transfection experiments.

miR-155 were markedly and specifically enriched in the culture medium compared to the intracellular compartment in amounts comparable to those from B-cells transfected with a single miRNA nucleotide sequence borne on an individual plasmid (Figure 3a). Likewise when B-cells were transfected with the plasmid carrying the nucleotide sequence of miR-150 and anti-miR-155 in tandem, they secreted each sncRNA in a range comparable to that of B-cells transfected with the plasmid carrying the nucleotide sequence of either miR-150 or anti-miR-155, respectively (Figure 3b). Together these results validate the notion that B-cells are very efficient at the extracellular export of newly-expressed sncRNAs leading to their rapid extracellular accumulation.

sncRNAs are enriched in EVs

A second and important feature of B-cells undergoing enforced expression and release of sncRNA molecules is

that sncRNAs are packaged in EVs.¹² Here we interrogated the efficiency at which this event occurs by determining whether two sncRNAs synthesized and released synchronously are apportioned equally in EVs, and by estimating the copy number/EV of the sncRNA cargo in different conditions.

EVs isolated from J558L cells cultured for 48 hours after transfection in medium containing normal fetal calf serum showed a high content of sncRNAs. The RQ values for miR-155 were markedly elevated in EVs released by B-cells transfected with a plasmid carrying one sncRNA and only slightly decreased in EVs released by B-cells transfected with a plasmid carrying two sncRNAs (Figure 4). At variance, the RQ values for miR-150 in EVs from B-cells transfected with a plasmid carrying two sncRNAs were reduced (90 versus 3,298 RQ) compared to reference EVs from B-cells transfected with a single sncRNA plasmid.

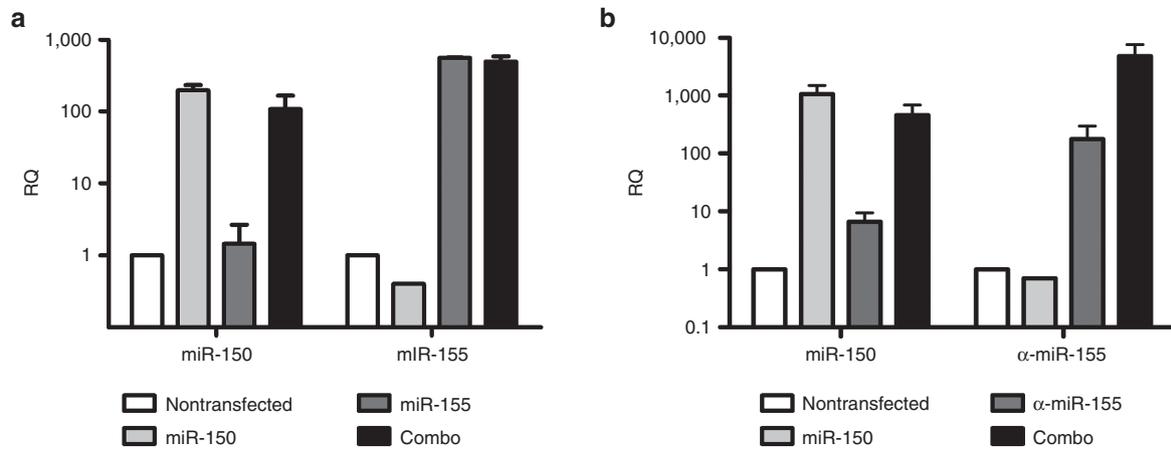


Figure 3 Detection of miR-150 and anti-miR-155 in the supernatant of programmed J558L cells. The supernatant of J558L cells transfected with a plasmid coding for miR-150 alone, miR-155 alone, or the combination of both (combo) was harvested 36 hours after transfection and total RNA extracted by the Zygem kit. cDNA was generated using RT-specific primers using Lifetech microRNA assay kit. Samples were preamplified and then subject to quantitative reverse transcription polymerase chain reaction amplification using RT-specific primers. Results refer to the mean ± SD of two independent transfection experiments.

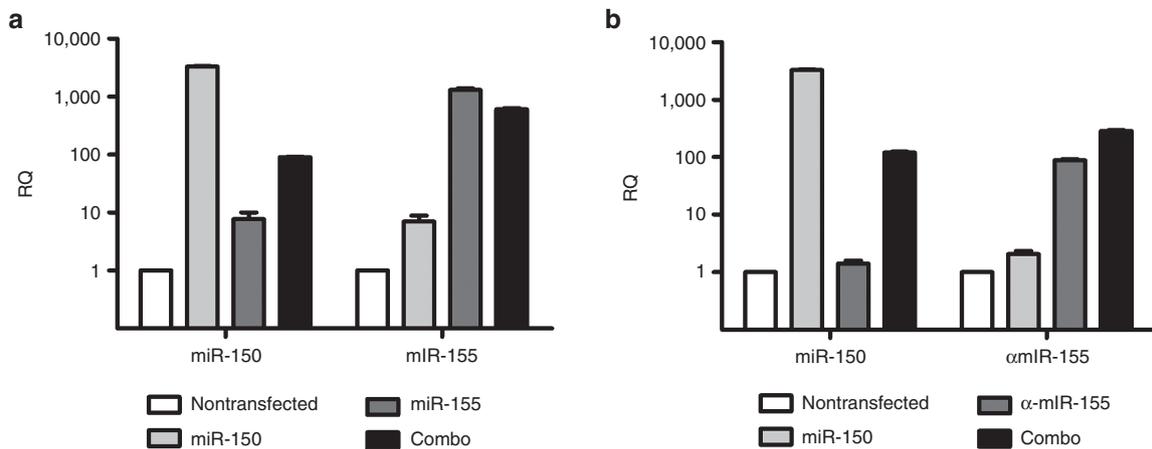


Figure 4 Relative quantitation of miR-150 and anti-miR-155 in EVs produced by programmed J558L cells. EVs were isolated from the supernatant of J558L cells transfected with a plasmid coding for (a) miR-150 alone, miR-155 alone or in combination (combo), and (b) miR-150 alone, anti-miR-155 alone, or in combination, 48 hours after transfection and culture in complete medium containing non exosome-depleted fetal calf serum. Total RNA was extracted by the Zygem kit. cDNA was generated using RT-specific primers using Lifetech microRNA assay kit. Samples were preamplified and then subject to quantitative reverse transcription polymerase chain reaction amplification using RT-specific primers. Results refer to the mean ± SD of two independent transfection experiments.

To obtain a more accurate estimate of the sncRNA content in EVs, experiments were repeated by analyzing the sncRNA content of EVs produced by programmed J558L cells cultured for 48 hours in commercially-available exosome-depleted medium. EVs were isolated and counted as described in Materials and Methods. Their average size ranged between 101 and 111 nm in EVs from J558L transfectants versus 106 nm in EVs from sham transfected J558L cells with minimal dispersity (**Supplementary Figure S1**). Their sncRNA content was assayed and quantified by RT-qPCR. From a total of 10^{11} EVs/sample reconstituted to 200 μ l, we extracted 0.3 and 0.2 mg/ml, respectively, suggesting that there is no substantial bias introduced by the transfection in the generation and protein content of EVs. Interestingly, the tetraspanin CD63, which is expressed in exosomes, is expressed in EVs released by B-cells transfected with a plasmid carrying two sncRNAs in relatively higher amounts than in EVs isolated from untransfected B-cells (**Figure 5**), suggesting a selective enrichment during the enforced sncRNA biogenesis, and EVs formation and release.

Next we determined the copy number of each sncRNA in EVs. **Figure 6** shows that each EV contains, on average, 3.6 copies of predetermined individual sncRNAs. Remarkably, we found comparable sncRNA copy numbers in EVs from B-cells programmed with dual or single sncRNA plasmid.

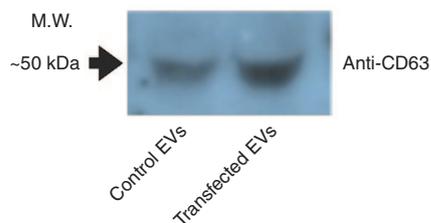


Figure 5 EVs produced by programmed J558L cells express CD63. EVs from J558L transfected with miR-150 and anti-miR-155 or untransfected (control) were processed for western blot analysis as described in Materials and Methods and probed with an antibody to CD63. The approximate m.w. was determined using a m.w. ladder (not shown).

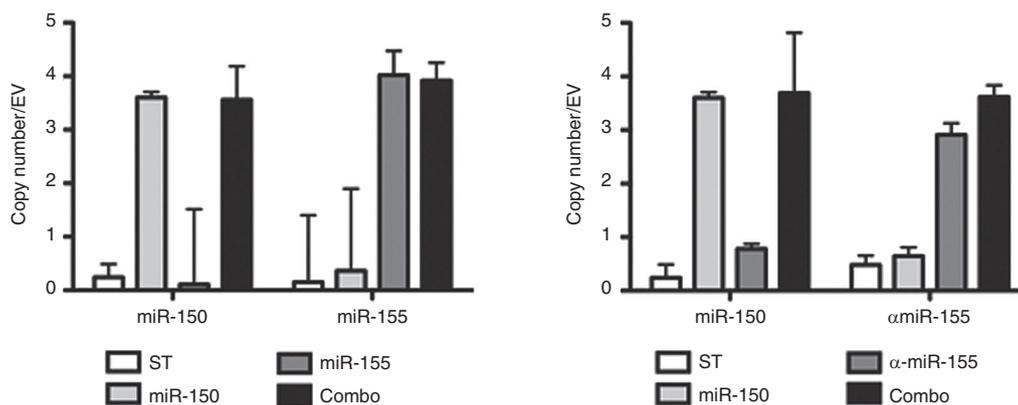


Figure 6 Copy number/EV quantitation of miR-150 and anti-miR-155 in EVs produced by programmed J558L cells. EVs were isolated from the supernatant of J558L cells transfected with a plasmid coding for miR-150 alone, miR-155 alone, or the combination of both (combo) 48 hours after transfection and culture in complete medium containing exosome-depleted fetal calf serum. Total RNA extraction and cDNA generation were performed as in Figure 4. Samples were pre-amplified and then subject to quantitative reverse transcription polymerase chain reaction amplification using RT-specific primers. Copy number/EV were calculated as described in materials and Methods. ST, Sham transfected. Results refer to the mean \pm SD of two replicate samples. Representative of two experiments with comparable results.

When compared to the constitutive content of miR-150 and miR-155 in EVs from sham-transfected J558L cells, the copy number was 15- and 25-fold higher, respectively. A copy number quantitation in EVs produced in exosome-depleted medium yielded on average a 30–50% increase, a result accounting for the decrease in B-cell-derived EVs by exosomes contained in commercial fetal calf serum (data not shown). Finally, we found that sncRNAs generated in tandem are effective at decreasing (~30%) target miRNA or at increasing cellular sncRNA content in transfected J558L cells (**Supplementary Figure S2**).

Discussion

We demonstrate that B-cells can be efficiently programmed for the synchronous biogenesis and secretion of multiple predetermined sncRNAs. We also found that the effective extracellular concentration of sncRNAs expressed in tandem in B-cells is markedly higher than in the intracellular compartment. Finally, we show that EVs released by B-cells programmed for the synchronous biogenesis and secretion of multiple predetermined sncRNAs are markedly enriched in sncRNA content.

Clinical applications of miRNA-based regulation of gene expression and disease may require the combined expression of multiple sncRNAs for therapeutic results. This can involve administering two or more miRNAs or a mixture of miRNAs, and anti-miRNAs. For example, miR-150 and miR-155 exist in B- and T-lymphocytes in reciprocal balancing regulation,^{16,17} necessitating a bimodal regulation. A multipronged approach may apply to other situations. For instance, two miRNAs may be an efficient method to target extracellular metabolic energetics and block cancer progression.¹⁸ Likewise, a multipronged miRNA-based approach may be necessary to simultaneously target complementary functions in cancer cells such as self renewal and pluripotency,¹⁹ and translation initiation.²⁰ Our data clearly show that this goal is attainable, in principle, using B-cells transfected with plasmid DNA purposely engineered for a multipronged effect. Since B-cells

transfected with plasmid DNA have already been used in a phase 1 trial²¹ showing no toxicity,²² B-cells programmed to secrete and release sncRNAs may be readily exploited for clinical translation. In addition to possessing a formidable and rapidly adjustable synthetic machinery, B-cells are capable of miRNA biogenesis^{23,24} and exosomes production.^{25,26}

Small (30–100 nm) microvesicles, exosomes, have surged to relevance as important intercellular messengers.^{27,28} EVs are released by B-lymphocytes,²⁶ T-lymphocytes,^{29,30} dendritic cells,^{31,32} and bone marrow-derived mesenchymal stem cells.³³ Exosomes also play a relevant role in cancer as intercellular messengers^{34,35} modulating cancer cells growth and metastasis,^{36–38} promoting therapy resistance,^{39,40} and orchestrating immune suppression.⁴¹

Exosomes are also regarded as vehicle for targeted gene therapy⁴² and cancer therapies.⁴³ However, the future of exosomes in therapeutic settings depends a priori on the efficiency with which a predetermined sncRNA cargo can be loaded onto exosomes during biogenesis. This has been found to vary depending on the cell type and the methods to generate the sncRNA cargo.⁴³ Of particular interest is a recent quantitative analysis to determine the stoichiometric relation between exosomes and their sncRNA content.⁴⁴ The study found that, regardless of the cell of origin, natural exosomes contain far less than one molecule of a given miRNA per exosome, even for the most abundant exosome preparations. This argues that spontaneously generated exosomes obey to a low occupancy/low miRNA concentration rule,⁴⁴ possibly the consequence of poor efficiency in either biogenesis or EV packaging. Our results show instead that the enforced and synchronous expression of multiple predetermined sncRNA in B-cells yields EVs much enriched for these sncRNAs. We calculated that EVs released by programmed B-cells contain, on average, 3.6 copies of specific sncRNA irrespective of whether the originating B-cells had been transfected with a dual or a single sncRNA plasmid. Thus, EVs released from programmed B-cells are many fold enriched in predetermined sncRNAs over the content of miRNA in natural exosomes,⁴⁴ a fact mirrored here by the 15–25-fold increase in copy number for miR-150 and miR-155 relative to the constitutive content of EVs from sham transfected J558L cells used as control. Since naturally exosomes carry a highly variable miRNA cargo with low content in specific sncRNA,⁴⁵ our method appears to resolve this potential problem in generation and production of EVs for therapeutic application. Importantly, we found that sncRNA generated in tandem are effective at regulating target miRNA or at increasing cellular sncRNA content in target cells as prerequisite for sncRNA therapeutic intervention.

The present demonstration relates to the mechanism of sncRNA cargo generation in vesicles destined to extracellular export. It is known that upon biogenesis sncRNA are packaged in late endosome multivesicular bodies.⁴⁶ A recent report showed that the artificial overexpression of a miRNA enriched its content in multivesicular bodies, and subsequently in exosomes, and is inversely proportional to the overexpression of miRNA target sequences⁴⁷ that can serve as a miRNA negative regulatory element by providing complementary binding sites.⁴⁸ Thus, sncRNA sorting to EVs may reflect the ability of the cell to dispose of sncRNA in excess

of their RNA cellular target. Accordingly, the production of sncRNA is expected to vary in different cell types. Although the exact mechanism of biogenesis and cargo packaging in our system remains to be elucidated, our study demonstrates that the rate of enforced biogenesis in B-cells is sufficient to outperform the ability of the cell to buffer the rate at which artificially expressed sncRNA are enriched in EVs.

In conclusion, we demonstrate that it is possible to program B-cells for the enforced biogenesis and release of multiple predetermined sncRNAs. The approach yields a greater sncRNA concentration in the extracellular than in the intracellular compartment suggesting an active transport mechanism. We also show that programmed B-cells release EVs with high copy number in predetermined sncRNAs. Collectively, B-cells programmed for the synchronous expression and secretion of multiple sncRNAs appear to be a viable candidate for multipronged translational applications to control disease or regulate immunity, and a step forward in the process of optimization and control in the production of EVs for miRNA-based therapies.

Materials and Methods

Plasmid constructs. Dual miRNA constructs containing miR-150/miR-155 and miR-150/anti-miR-155 were synthesized with unique ends SgfI/XhoI by Integrated DNA Technologies (IDT, Coralville, IA). Constructs were cloned into the pCMVmir (Origene, Rockville, MD) expression vector by digesting with SgfI and XhoI, and subsequent ligation of the insert into the pCMVmir vector. The ligation mixture was transformed into DH5 α competent cells. Transformed cells were plated, and clones were selected and grown overnight at 37 °C. DNA was extracted with Promega Wizard Plus SV Minipreps DNA Purification System (Promega, Madison WI). The resultant plasmids were termed pCMV mir150+mir155 and pCMV mir150+mir α 155. The clone inserts were verified via sequencing and stored at –20 °C until transfection. Single miRNA constructs containing miR-150 or miR-155 were generated through excision from the dual miRNA constructs by digestion and ligation using unique restriction sites (SgfI/MluI or NotI-XhoI) within the minigene to yield pCMV miR-150, pCMV miR-155, and pCMV anti-miR-155, respectively. The correctness of each plasmid construct was verified by sequencing.

Cell culture and transfection. J558L mouse B-cell myeloma cells were grown in suspension in cRPMI with 10% fetal bovine serum (FBS). Cells were grown to 80% confluence. 2 \times 10⁶ cells were transfected with 1 μ g of pCMVmiR plasmid utilizing the Lonza VACA-1003 transfection kit V and Nucleofector 2b device (Lonza, Walkersville, MD). Cells were allowed to recover in a T25 flask upright at 37 °C with 5% CO₂ for 48 hours. In experiments in which sncRNA copy number was determined, transfected B-cells were cultured in EXO-FBS-50A-1 exosome-depleted FBS (Exo-FBS, Systems Biosciences, Mountain View, CA).

EV isolation. Forty-eight hours post-transfection, 200 μ l of culture supernatant were collected and incubated with 200 μ l

of Total Exosome Isolation solution (Life Technologies, Carlsbad, CA) at room temperature for 1 hour. The EV containing mixture was spun at 16,000 RPM at 4 °C for 1 hour. The resultant EV pellet was resuspended in 50 µl of PBS at room temperature and stored in 1.5 ml Eppendorf tubes at –20 °C until use. EVs isolated from untransfected or sham transfected (electroporated only) J558L cells served as a control.

Nanoparticle tracking analysis. The number of vesicles recovered was determined by nanoparticle tracking analysis on a NanoSight LM-10HS equipped with a 405 nm laser (NanoSight, Wiltshire, UK) that was calibrated with polystyrene latex microbeads at 100 and 200 nm prior to analysis. Resuspended vesicles were diluted 1:50 with PBS to achieve between 20–100 objects per frame. EVs were manually injected into the sample chamber at ambient temperature. Each sample was measured in triplicate at camera setting 14 with acquisition time of 30 seconds and detection threshold setting of 7. At least 200 completed tracks were analyzed per video. The nanoparticle tracking analysis analytical software version 2.3 was used for capturing and analyzing the data.

Western blot. EVs were lysed in radioimmunoprecipitation assay buffer (1% NP40, 0.5% deoxycholate, 0.1% sodium dodecyl sulfate in Tris-buffered saline). Protein concentration was determined by NanoDrop spectrophotometer. Fifteen micrograms of proteins of each sample were separated in 4–20% acrylamide/bisacrylamide gel and transferred to a polyvinylidene difluoride membrane using Trans-Blot Turbo system (Bio-Rad) for 3 minutes.

mini-TGX protocol. After washing in TBST, the membrane was incubated with an anti-CD63 monoclonal antibody (5 µg ml⁻¹) (abcam) (the kind gift of Dr. Johnny Akers) overnight at 4 °C on a rocker. After washing in TBST, the bound antibody was revealed using goat antibodies to mouse Ig conjugated to horseradish peroxidase (5 µg ml⁻¹) (Bio-Rad, Hercules, CA). The blot was developed with electrochemiluminescence substrate and exposed to X-ray film for 3 minutes.

RNA extraction. 5 × 10⁵ transfected or untransfected J558L cells, and 1 ml of culture supernatant, were collected for RNA extraction using ZYGEM RNAtissue Plus System (Zygem, Hamilton, NZ) according to the manufacturer's protocol. RNA from cell supernatant (200 µl) was extracted with the Qiagen miRNeasy Serum/Plasma kit following the manufacturer's protocol. EVs extraction was performed using the ZYGEM RNAtissue Plus System.

Small RNA Taqman. cDNA was generated from intracellular, extracellular and exosome miRNA with Taqman small RNA assays. Input RNA was normalized to 100 ng/sample for intracellular and exosome RNA, and to 25 ng/sample for extracellular miRNA. Taqman MicroRNA Reverse Transcription Kit was utilized for all samples per manufacturer's instructions. Cycling conditions for qPCR were: 40 cycles, 96 °C denature 30 seconds, 60 °C anneal/extension 30 seconds. Results are expressed as RQ (relative quantity of sample) that was calculated using the formula: $\text{Relative quantity}_{\text{target}} = \frac{E_{\text{target}}}{C_q(\text{control}) - C_q(\text{treatment})}$. Abbreviations: E = Efficiency

of primer set; C_q (control) = Average C_q for the control or untreated sample; C_q (treatment) = Average C_q for treated sample; Target = The gene of interest or reference gene.

Copy number determination. To determine the copy number of miR-150, miR-155, and anti-miR-150, samples normalized at 100 ng cDNA/reaction were run concomitantly with a standard curve constructed with known amounts (100–0.01 ng) of each short noncoding RNA cDNA. The endogenous control standard curve was constructed using known amounts (100–0.01 ng) of snoRNA202 cDNA of all the targets (Applied Biosystems snoRNA202—assay No. 001232—specific reverse transcription primers). Samples were run in duplicate. Relative expression was determined by the Ct value of test samples versus the endogenous control. Once the amount (ng) of specific target was determined, the copy number present in each reaction was calculated using the following formula: $(\text{ng} \times 6.0223 \times 10^{23}) / (\text{number of nucleotides} \times 1.0 \times 10^9 \times 650)$ as indicated in <http://www.uic.edu/depts/rrc/cgf/realtime/stdcurve.html>. Copy number/EV determination was calculated as follows: (total copy number/No. EVs sample).

Supplementary Material

Figure S1. NanoSight analysis of particle size and distribution in the supernatant of J558L transfectants.

Figure S2. sncRNA produced in J558L cells by dual transfection regulate the endogeneous content of sncRNA.

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