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Chemical Transfection of Dye-Conjugated MicroRNA Precursors for MicroRNA Functional Analysis of M2 Macrophages

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ABSTRACT

MicroRNAs (miRNAs) are short noncoding ribonucleic acids known to affect gene expression at the translational level and there is mounting evidence that miRNAs play a role in the function of tumor-associated macrophages (TAMs). To aid the functional analyses of miRNAs in an in-vitro model of TAMs known as M2 macrophages, a transfection method to introduce artificial miRNA constructs or miRNA molecules into primary human monocytes is needed. Unlike differentiated macrophages or dendritic cells, undifferentiated primary human monocytes have been known to show resistance to lentiviral transduction. To circumvent this challenge, other techniques such as electroporation and chemical transfection have been used in other applications to deliver small gene constructs into human monocytes. To date, no studies have compared these two methods objectively to evaluate their suitability in the miRNA functional analysis of M2 macrophages. Of the methods tested, the electroporation of miRNA-construct containing plasmids and the chemical transfection of miRNA precursor molecules are the most efficient approaches. The use of a silencer siRNA labeling kit (Ambion) to conjugate Cy 3 fluorescence dyes to the precursor molecules allowed the isolation of successfully transfected cells with fluorescence-activated cell sorting. The chemical transfection of these dye-conjugated miRNA precursors yield an efficiency of $37.5 \pm 0.6\%$ and a cell viability of $74 \pm 1\%$. RNA purified from the isolated cells demonstrated good quality, and was fit for subsequent mRNA expression qPCR analysis. While electroporation of plasmids containing miRNA constructs yield transfection efficiencies comparable to chemical transfection of miRNA precursors, these electroporated primary monocytes seemed to have lost their potential for differentiation. Among the most common methods of transfection, the chemical transfection of dye-conjugated miRNA precursors was determined to be the best-suited approach for the functional analysis of M2 macrophages. J. Cell. Biochem. 113: 1714–1723, 2012. © 2011 Wiley Periodicals, Inc.

Tumor-associated macrophages (TAMs) are increasingly implicated in the pathology of cancer proliferation and metastasis [Tjiu et al., 2009; Liu et al., 2011; Squadrito and De Palma, 2011; Wang et al., 2011]. TAMs result from the recruitment of peripherally circulating monocytes into tumor sites and the subsequent activation of these monocytes by cytokines present in the tumor microenvironment. Commonly discussed in the pathogenesis of cancer are two classes of TAMs: the classically activated M1 macrophages and the alternatively activated M2 macrophages. M1 macrophages are activated by Th1 cytokines, such as interferon-gamma (IFNg), or microbial triggers, such as lipopolysaccharide (LPS). They enhance microbicidal activity, intensify cell-mediated

immunity and constitute the body's own immune reaction to cancer. The M2 macrophages, on the other hand, are activated by Th2 cytokines such as interleukin 4 (IL4), interleukin 13 (IL13), or macrophage colony-stimulating factor (MCSF). Unlike M1 macrophages, the M2 macrophages function to tune inflammatory responses, scavenge debris, and participate in angiogenesis, tissue remodeling, and repair. Due to their immunosuppressive traits, M2 macrophages are generally known to be counter-productive in the body's fight against cancer as they contribute to a tumor microenvironment that is conducive for tumor growth and metastasis.

MicroRNAs (miRNAs) are short noncoding ribonucleic acids that are known to affect gene expression at the translational level [Dai and Ahmed, 2011]. There is increasing interest in the role miRNAs play in the differentiation of M2 macrophages [He et al., 2009; Martinez-Nunez et al., 2011]. For instance, the under-expression of hsa-miR-155 in M2 macrophages has recently been shown to modulate the signaling pathway of interleukin 13, an important pro-M2 cytokine [Martinez-Nunez et al., 2011]. To aid the functional analyses of miRNAs in tumor-associated macrophages, the development of an efficient method to deliver artificial miRNA constructs or miRNA molecules into primary human monocytes would be beneficial. Since the goal is to study the miRNAs' function in M2 macrophages, including their role in driving the differentiation process, it is important that a transfection method is able to target uncommitted, freshly isolated peripheral monocytes.

Unlike differentiated macrophages and dendritic cells, primary human monocytes have been known to show resistance to lentiviral transduction [Neil et al., 2001]. To circumvent the low lentiviral transduction efficiencies, other techniques such as electroporation and chemical transfection have been used to introduce small gene constructs into human monocytes. Table I is a summary of recent transfection attempts on cells belonging to the monocyte-macrophage lineage. Two incidences of successful transfection of freshly isolated (day 0) primary monocytes have been described. Ponsaerts et al. [2002] used electroporation to drive messenger RNA molecules into primary monocytes, whereas Martinez-Nunez et al. [2011] used chemical transfection to deliver anti-miRNA oligonucleotides. These two methods can be improved as they lacked a selection system to isolate only the successfully transfected cells for further analysis. Additionally, it was unclear if these two methods affected the primary monocytes' potential to differentiate into M2 macrophages.

In order to address both challenges, electroporation and chemical transfection were compared to determine the best-suited option for this application. These two approaches were tested for its ability to drive either dye-conjugated miRNA precursors or miRNA-constructs containing plasmids into primary monocytes.

To determine "suitability" of a method for functional analysis of miRNA in M2 macrophages, each method was evaluated based on four criteria:

(1) Transfection efficiency (>20%)
(2) Fluorescence reporter system to allow selection
(3) Biological activity
(4) Demonstrate potential to differentiate into M2 macrophages after transfection

While plasmids containing microRNA constructs commonly come with GFP selection markers, miRNA precursors with flurochrome markers are not readily available. To establish a reporter system for the transfection of miRNA precursor molecules, the precursor molecules were conjugated with Cyanine3 (Cy3) dye using the silencer siRNA labeling kit (Ambion). Since this labeling technique was originally used for labeling small interfering RNAs (siRNAs), which are structurally and functionally similar to miRNAs, the use of the kit was expected to be compatible with miRNAs. As an added precaution, it was later demonstrated that the dye conjugation has no effects on the biological activity of the miRNA precursors.

The approaches that showed acceptable transfection efficiencies were then tested for the fourth criterion. This was done post-transfection by treating the cells with IL4 and MCSF (pro-M2 macrophages stimulus) and analyzing their surface expression of CD206 (mannose receptor; widely accepted cell surface marker of M2 macrophages). The increased expression of CD206 indicated the differentiation of monocytes to M2 macrophages and implied that the internal machinery that permits differentiation remained intact after transfection. These four criteria describe what researchers studying tumor-associated macrophages would require for the functional analysis of miRNA in TAMs.

Using hsa-miR-511 as an example, the feasibility of introducing a relevant microRNA into primary human monocytes was demonstrated. Hsa-miR-511 is a microRNA located in the fifth intron of the CD206 gene and co-expressed with CD206, which M2 macrophages express in abundance.

TABLE I. Summary of Comparison Between Methods

Approach	Target transfectants	Plasmids/RNA oligonucleotides	Citations
Lentiviral transduction	4 Day old human primary monocytes	Simian lentivirus-derived vector	Muhlebach et al. [2005]
Lentiviral transduction	0 Day old MCSF differentiated macrophages	HIV-based lentivirus	Leyva et al. [2011]
Electroporation	0 Day old human primary monocytes	Messenger RNA	Ponsaerts et al. [2002]
Electroporation	6 Day old monocyte-derived dendritic cells	siRNA (small interfering RNA)	Prechtel et al. [2006]
Electroporation	3 Day old monocyte-derived dendritic cells	Plasmid DNA	Landi et al. [2007]
Electroporation	6 Day old monocyte-derived dendritic cells	Plasmid DNA	Lenz et al. [2003]
Chemical transfection	1 Day old human primary monocytes	miRNA oligonucleotides	He et al. [2009]
Chemical transfection	5 Day old monocyte-derived dendritic cells	Anti-miRNA oligonucleotides	Martinez-Nunez et al. [2009]
Chemical transfection	0 Day old human primary monocytes	Anti-miRNA oligonucleotides	Martinez-Nunez et al. [2011]

RESULTS

IN VITRO GENERATION OF M2 MACROPHAGES

The in vitro generation of M2 macrophages through the alternative activation of primary monocytes was achieved via incubation with pro-M2 cytokines such as IL4 or MCSF, as previously described [Verreck et al., 2004; Martinez et al., 2006; Puig-Kroger et al., 2009]. These results were reproduced using 100 ng/ml IL4, 50 ng/ml MCSF or a mixture of both. CD206 (also known as *MRC1*, mannose receptor 1) is a specific surface marker of M2 macrophages [Mantovani et al., 2002] and is used in these experiments as an indicator of M2 macrophage differentiation. Cytometric analysis was carried out 48 h later to quantify the surface expression of CD206 showed that the percentage of CD206 expressing M2 macrophages were 35.9% in untreated wells, 22.6% in IFNg-treated wells, 96.3% in IL4-treated wells, 59.0% in MCSF-treated wells, and 98.4% in wells treated with a combination of IL4 and MCSF (Supplemental Fig. 1). By qRT-PCR, the expression levels of hsa-miR-511 were measured to be higher in IL4 or MCSF treated M2 macrophages compared to IFN-g treated M1 macrophages or untreated control cells.

COMPARISON OF TRANSFECTION EFFICIENCIES AND CELL VIABILITIES

Figure 1 shows a representative experiment in which each transfection method was used to deliver hsa-miR-511 into the cells. The transfection efficiencies were compared between lentiviral transduction, electroporation, and chemical transfection. The transfection efficiencies were represented by the percentage of cells that showed fluorescence intensities above those of non-transfected cells. Figure 2A summarizes the transfection efficiencies of the different methods. Efficiency for lentiviral transduction was $1.7 \pm 0.3\%$. Increase of the viral titer to $5\times$ with the addition of polybrene during lentiviral transduction only modestly improved the transfection efficiency to 2.2% (Supplemental Fig. 2). Electroporation demonstrated best efficiency when it was being used with miRNA-construct containing plasmids, and resulted in a transfection efficiency of $42.8 \pm 3.6\%$, as compared to $0.9 \pm 0.1\%$ when used with miRNA precursors. Chemical transfection on the other hand, demonstrated the best efficiency when it was being used with miRNA precursors, and resulted in a transfection efficiency of $37.5 \pm 0.6\%$, as compared to $2.6 \pm 0.5\%$ when used with plasmids.

Viability staining with 7-aminoactinomycin D (7-AAD) was performed to evaluate cell mortality as a result of the transfections (Fig. 2B). The viability was measured to be $40.8 \pm 2.6\%$ for lentiviral transduced cells, $35.6 \pm 2.1\%$ for cells electroporated with plasmids, $56.6 \pm 1.4\%$ for cells electroporated with miRNA precursors, $38.3 \pm 7.7\%$ for cells chemically transfected with plasmids, and $73.8 \pm 1.0\%$ for cells chemically transfected with miRNA precursors.

The two methods that showed acceptable transfection efficiencies were electroporation of plasmids and chemical transfection of miRNA precursors. However, between these two approaches, the electroporation of plasmids also produced the highest mortality.

POTENTIAL FOR DIFFERENTIATION WAS PRESERVED IN CELLS CHEMICALLY TRANSFECTED WITH miRNA PRECURSORS

Since the transfection processes could potentially change the characteristics of the cells, the capability of transfected cells to differentiate was evaluated. Pro-M2 cytokines (IL4 and MCSF) were used to stimulate transfected cells to transform into M2 macrophages in the same manner as in previous experiments. If the potential for differentiation remained intact, cells would then respond to the stimulation, and an increase in the expression of CD206 receptors could be observed among the transfected cell population (represented by the percentage of successfully transfected cells that were positive for CD206 receptors). As shown in Figure 3A, the addition of IL4 and MCSF after plating to chemically transfected cells increased the proportion of CD206 expressing cells to $88.9 \pm 1.2\%$, a significant increase compared to $10.6 \pm 0.7\%$ in the unstimulated cell population. For the electroporated cells, the proportion of CD206 expressing cells remained at $9.5 \pm 3.5\%$ even with the addition of pro-M2 cytokines.

The lack of differentiation was also evident when the cells were directly visualized with light microscopy (Fig. 3B). Normally, as monocytic cells differentiate into macrophages, they form an elongated shape with prodosomes protruding at both ends [Veale et al., 2011]. As shown in Figure 3B, cells chemically transfected with miRNA precursors displayed the elongated morphological appearance after they were stimulated with IL4 and MCSF for 48 h. Monocytes electroporated with plasmids on the other hand retained their round shapes after cytokine stimulation, likely reflecting their lack of differentiation.

CHEMICAL TRANSFECTION OF miR-1 PRECURSORS RESULTED IN THEIR PREDICTED EFFECTS

Chemical transfection of dye-conjugated miRNA precursors allowed the isolation of successfully transfected cells using fluorescence-activated cell sorting (FACS) so that they could be further analyzed. Since the targeted effects of hsa-miR-511 have not yet been studied, the transfection of hsa-miR-1 was used instead to demonstrate the miRNA activity of the transfected precursor molecules. Twifilin (PTK9) is a validated target of hsa-miR-1, and the knockdown happens at the mRNA level [Lim et al., 2005]. The inverse relationship between hsa-miR-1 expression and the expression level of PTK9 provided a convenient way to prove the activity of the miRNA precursor molecules once they were transfected into the cells. Pre-miR-1-Cy3 and its scrambled equivalent, NegCtrl#1-Cy3 were transfected into primary monocytes. Successfully transfected cells were sorted with FACS and then collected for RNA purification. The purification yielded RNA of high quality (RIN of 9.1–9.3). When qRT-PCR was performed, the pre-miR-1-Cy3 transfected cells displayed a 42% knock down of PTK9 compared to the NegCtrl#1 transfected cells (Fig. 4).

VIRAL TRANSDUCTION REPLICATION CAPABLE MONOCYTIC CELL LINES U937 AND THP-1

Measured by the proportion of GFP positive cells, the average transduction efficiencies were 0.37% for primary monocytes, 0.3% for THP-1 cells and 17.9% for U936 cells (Supplemental Fig. 2). Increasing the viral titer to $5\times$ increased the transfection efficiency
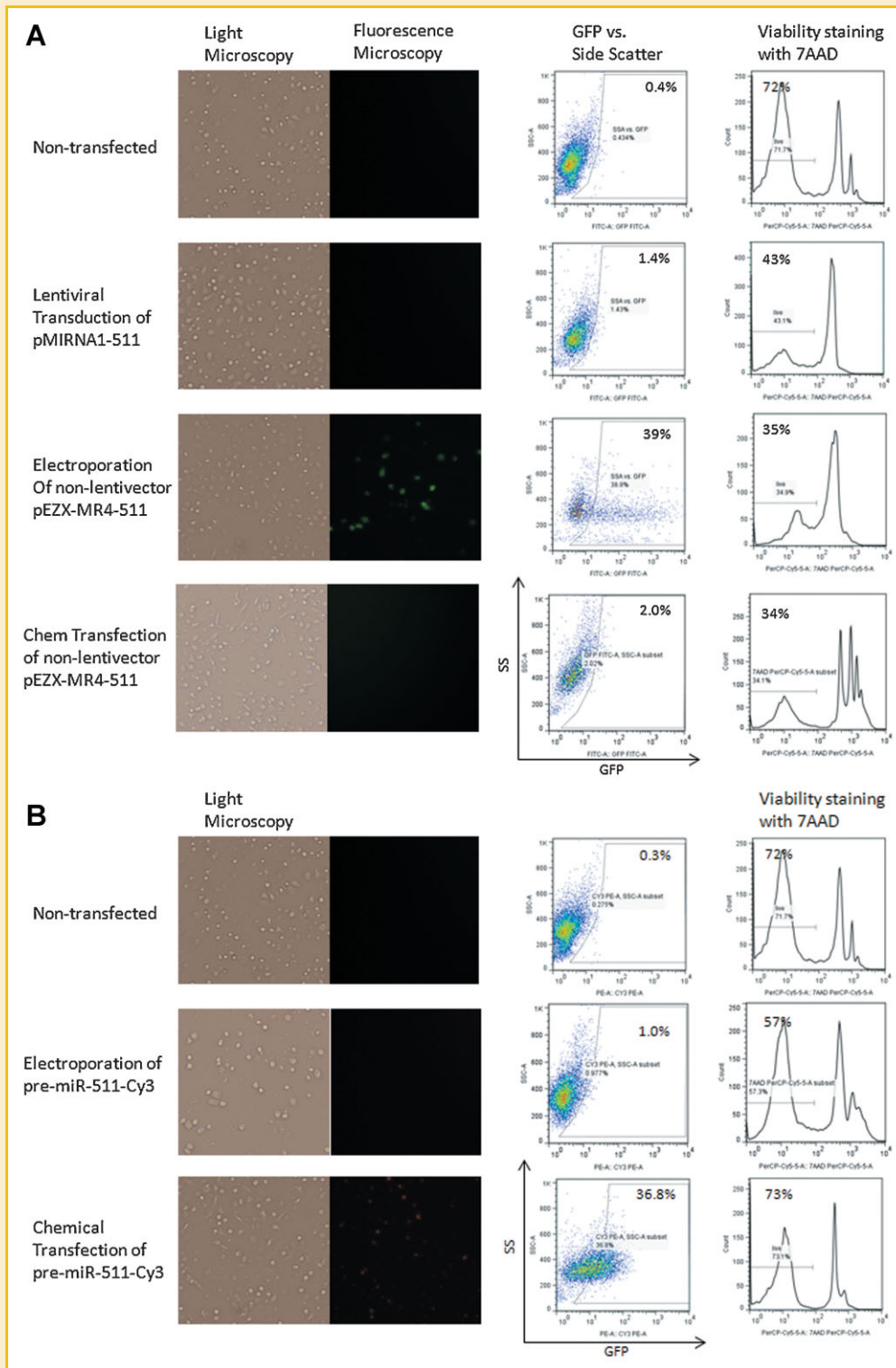
Fig. 1. Comparison of methods for the transfection of primary monocytes. Each transfection to over-express hsa-miR-511 was attempted in biological triplicates. A representative set of results was displayed in this figure (see Fig. 2 for more comprehensive results). Successful transfection was observed by fluorescence microscopy in both the electroporation and chemical transfection approach. The transfection efficiencies as indicated by the percentage of fluorescence positive cells that were measured by cytometry. Transfection is most efficient in the electroporation of pEZX-MR4 plasmids and the chemical transfection of microRNA precursors. The electroporation of pEZX-MR4 plasmids and the chemical transfection of microRNA precursors each show a substantial increase in the proportion of cells displaying fluorescence signals (39% and 37%, respectively). The lentiviral approach, electroporation of microRNA precursors, and the chemical transfection of pEZX-MR4 plasmids showed transfection efficiencies of about 1–2%, which were not at a level that is acceptable for functional analysis. Viability staining with 7-AAD performed on the transfected cells showed that chemical transfection of miRNA precursors led to the least cell mortality, while electroporation of pEZX-MR4 plasmids caused a substantial loss of cell viability.
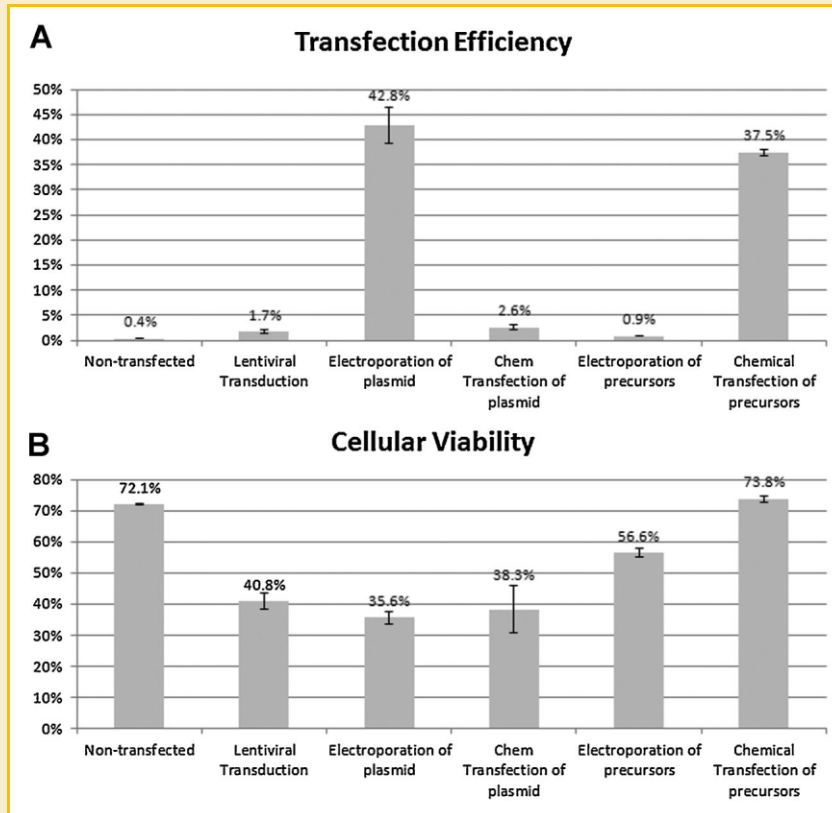
Fig. 2. Summary of the comparison of transfection efficiencies. Each transfection to over–express hsa–miR–511 was attempted in biological triplicates and the results of the transfection were as displayed in the figure. The error bars represent standard deviations. A: Transfection efficiencies were $1.7 \pm 0.3\%$, $43 \pm 4\%$, $2.6 \pm 0.5\%$, $0.9 \pm 0.1\%$ and $37.5 \pm 0.6\%$ for lentiviral transduction, electroporation of plasmids, chemical transfection of plasmids, electroporation of miRNA precursors, and chemical transfection of miRNA precursors, respectively. B: Cell viability by 7–AAD staining were $41 \pm 3\%$, $36 \pm 2\%$, $38 \pm 7\%$, $57 \pm 1\%$, and $74 \pm 1\%$ for lentiviral transduction, electroporation of plasmids, chemical transfection of plasmids, electroporation of miRNA precursors, and chemical transfection of miRNA precursors, respectively.

of THP-1 cells to 25%. As cell lines continued to replicate after lentiviral transduction, repeat enrichment yielded stably transduced cell populations (data not shown). These results show that while viral transduction is a viable method for the immortalized cell lines, transduction of primary monocytes yield a transfection efficiency that is too low for the functional analyses.

DISCUSSION

Recently, the differential expression of microRNAs has been found to serve an important function in the context of tumor-associated macrophage differentiation. To study the roles that these miRNAs play, there needs to be a method of delivering microRNAs into primary monocytes. This study determines that the method which is best suited to study miRNA function in M2 macrophages is the chemical transfection of dye-conjugated miRNA precursors.

When the transfection efficiencies were compared, the methods that fared well were the electroporation of human monocytes with nonlentivector plasmid DNA and the chemical transfection of dye-conjugated miRNA precursors. As expected, lentiviral transduction was inefficient. Previous studies on HIV-1 infection in primary

monocytes have elucidated that the apparent low transduction efficiency was due to the post-infection restriction of viral gene expression. The deficiency of host factors necessary for the transactivation of the LTR promoter in the vector resulted in an impaired reverse transcription, nuclear import, and integration of viral DNA [Neil et al., 2001; Triques and Stevenson, 2004; Dong et al., 2009]. These mechanisms also explained the lower efficiencies when lentivector plasmids were electroporated into human monocytes compared to nonlentivector plasmids (data not shown), since the same host factors were needed to express lentivector plasmids regardless of the mode of entry. Despite the low efficiencies observed in the lentiviral transduction of primary monocytes, there had been some development in this area. By altering the vector composition, Muhlebach et al. [2005] was able to transfect 4-day-old monocytes with simian lentiviral vector PBj. Although the success currently only extends to 4-day-old monocytes, there is potential in this method to be useful in the future.

Although both electroporation and chemical transfection are highly efficient, only the latter responded to IL4 and MCSF stimulation and differentiate into M2 macrophages. CD206 is a surface protein that serves functions that reflect the purpose of M2 macrophages, such as phagocytosis, intracellular signaling, and immunomodulation [Gazi and Martinez-Pomares, 2009]. The
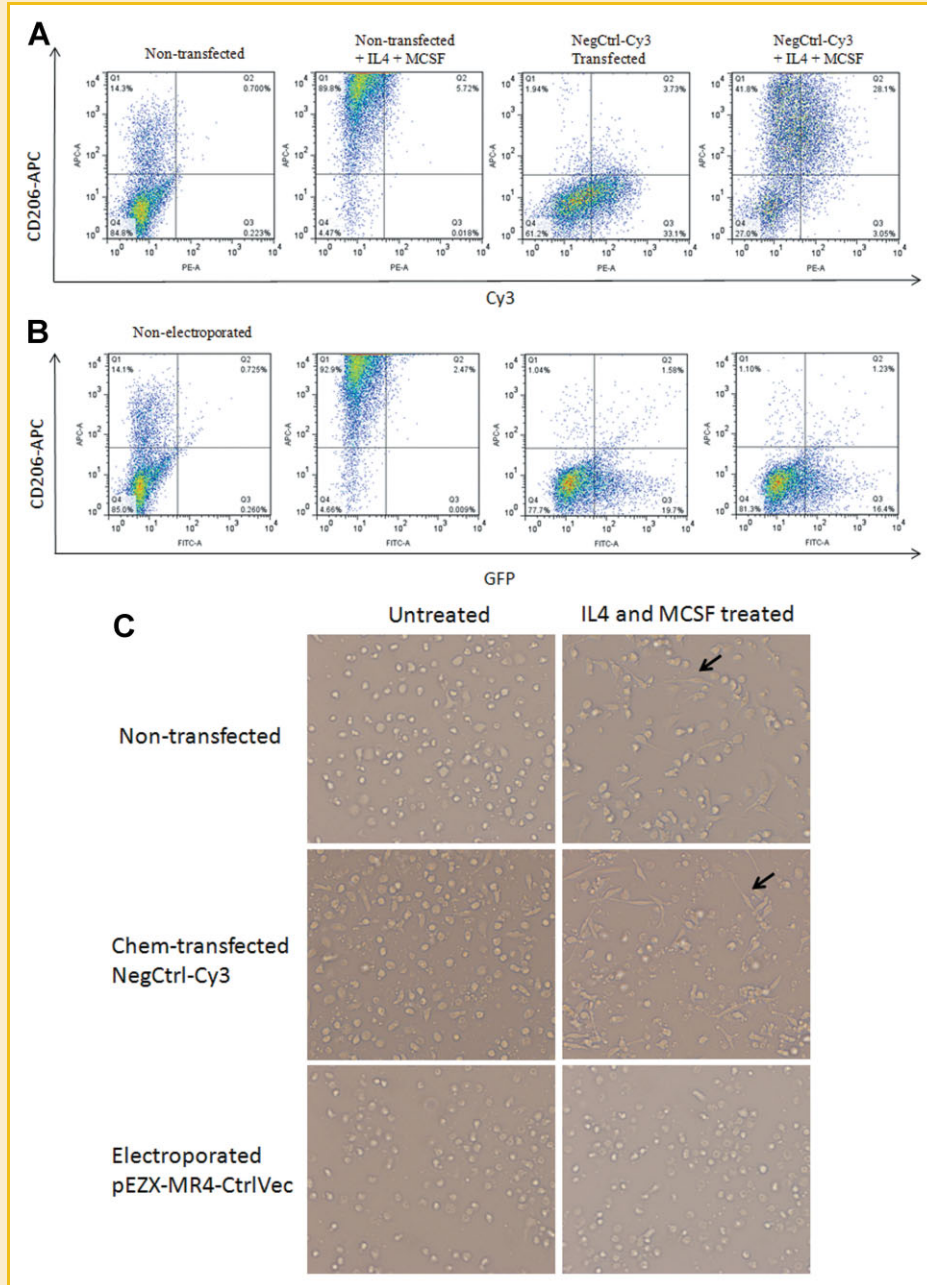
Fig. 3. Post-transfection stimulation of cells with pro-M2 cytokines. The two methods that demonstrated high transfection efficiencies were tested for the monocytes potential to differentiate post-transfection. At 1 h after transfection, pro-M2 cytokines IL4 and MCSF were added to the wells at 100 ng/ml and 50 ng/μl, respectively. Expression of CD206 were analyzed 48 h later. Each transfection and treatment was done in triplicates. A: Post-transfection stimulation results were displayed. The x-axis corresponded to Cy3 fluorescence intensity while the y-axis indicated expression of CD206, whose antibodies were conjugated to Allophycyanin for detection. Note that only transfected cells display increase in Cy3 fluorescence. In the fourth cytometric plot from the left showed cells stimulated with pro-M2 cytokines. The top right quadrant of the plot corresponded to cells that were both transfected and transformed into CD206-expressing M2 macrophages. The addition of IL4 and MCSF after plating to chemically transfected cells increased the proportion of CD206 expressing cells analyzed 48 h later to 88.9 ± 1.2%, a significant increase compared to 10.6 ± 0.7% in the unstimulated cell population. B: Post-transfection stimulation performed for cells electroporated with pEZX-MR4 plasmids as shown. For the successfully electroporated cells, the proportion of CD206 expressing cells remained at 9.5 ± 3.5% even with the addition of pro-M2 cytokines. In the unstimulated cell population, this proportion was 10.1 ± 1.5%. Note that unlike chemical transfection, there was little expression of CD206 surface markers in the electroporated cells even after the addition of pro-M2 cytokines. C: Light microscopy was also used to verify macrophage differentiation. Normally, as monocytic cells differentiate into macrophages, they form an elongated shape with prodosomes protruding at both ends [Veale et al., 2011]. As shown, cells chemically transfected with miRNA precursors display the elongated morphological appearance after they were stimulated with IL4 and MCSF for 48 h. Monocytes electroporated with plasmids on the other hand retained their round shapes after cytokine stimulation, likely reflecting their lack of differentiation.
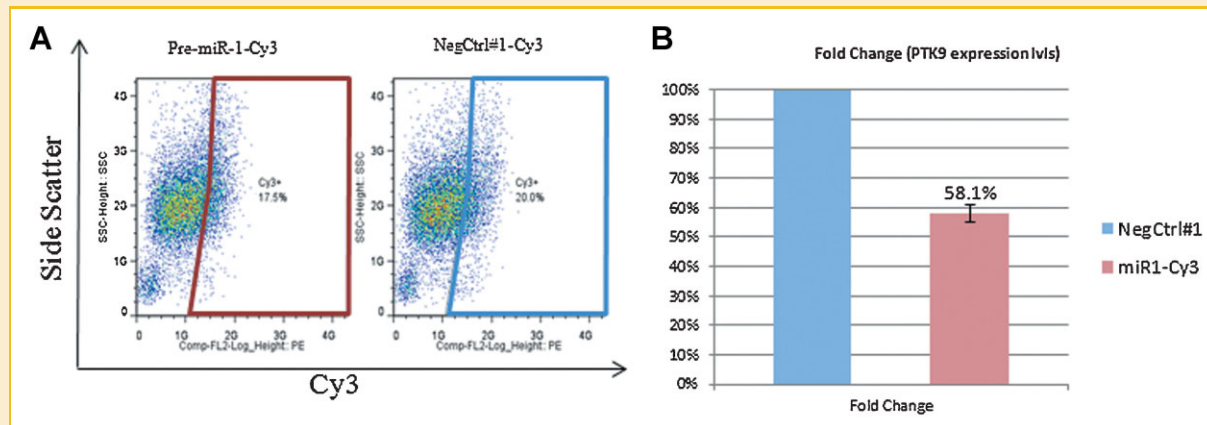
Fig. 4. Forty-two percent knock down of PTK9 by miR-1. To demonstrate that miRNA precursors that were chemically transfected into primary monocytes, Cy3-conjugated miR-1 precursor molecules (pre-miR-1-Cy3) were transfected. Hsa-miR-1 has been shown in human cells to cause a decrease in the level of PTK9 mRNA levels. A: Transfected cells that expressing high Cy3 intensities were sorted out using FACS. B: qRT-PCR was used to measure mRNA levels of PTK9. A 41.9 ± 0.3% knock down of PTK9 was observed in pre-miR-1-Cy3 transfected monocytes compared to NegCtrl#1-Cy3 (scrambled control for miRNA precursors).

inability of the electroporated cells to express CD206 after stimulation with pro-M2 cytokines indicated a change in the internal biology involved in the differentiation; therefore, the study of the biological effects of the miRNA is less optimal in this case. The question as to why electroporation could affect the differentiation of M2 macrophages is a curious one. It was observed that the approaches to drive plasmid DNA into the cells that were tested in this study seemed to result in drastically lower cell viability, even in cases when the transfections were not efficient. It is plausible that once transfected into the cells, the plasmid DNA were sensed by inflammasomes within the cells and led to the classical activation of macrophages, as described by Muruve et al. [2008]. This process along with higher cellular mortality could be few of many reasons that had crippled the monocytes' ability to differentiate.

Since miRNA precursors for chemical transfection available through commercial vendors do not carry a reporter system, miRNA precursors were conjugated with cy-3 dyes first. A further validation step was therefore needed to ensure that the conjugation did not alter the biological activity of the miRNA precursors. PTK9 (twinfillin) was a target of hsa-miR-1 and the down-regulation occurred at the messenger RNA level, as described previously [Lim et al., 2005]. If the inverse relationship between hsa-miR-1 and its target PTK9 could be seen in the chemical transfection approach, the biological activity of transfected hsa-miR-1 precursor can then be extended to other miRNAs. A 42% knockdown of PTK9 was seen in miR-1 transfected cells. Although a higher knockdown had been reported with other cell types, this is likely due to the difference

in the potency of hsa-miR-1 as it acts within different cell types. Since hsa-miR-1 is a brain and muscle tissue specific miRNA [Lim et al., 2005], the knockdown of PTK9 would reasonably be more pronounced in these tissues. This is also a demonstration of the need for the isolation of transfected cells prior to analysis. The efficiency of chemical transfection was only 38% after optimization, and the increase in the contrast provided by the isolation step allowed less sensitive miRNA targets to be studied.

A high quality of RNA (RIN of 9.1–9.3) was achieved from the isolated cells. The lack of RNA degradation meant that the transfected cells were healthy, and reliable RNA analysis can be done. Although only real-time qPCR was demonstrated here, because of the high quality of RNA, amplification methods can be used to allow higher throughput techniques such as microArray to be employed [Clement-Ziza et al., 2009].

CONCLUSIONS

In summary, the results of this study are as shown in Table II. Chemical transfection is the only method that has fulfilled all four criteria. Based on the experiments presented in this study, chemical transfection of dye-conjugated miRNA precursors is the method that will most likely suit the needs of researchers in the field, especially those who are studying the functions of miRNAs in the context of M2 macrophage differentiation.

TABLE II. Summary of Comparison Between Methods

	Transfection efficiency	Reporting system	Biological activity	Ability to differentiate
Lentiviral transduction	×	✔	N/A	N/A
Electroporation w/plasmid constructs	✔	✔	N/A	×
Electroporation w/precursors	×	✔	N/A	N/A
Chemical transfection w/plasmid constructs	×	✔	N/A	N/A
Chemical transfection w/precursors	✔	✔	✔	✔

METHODS

CELL CULTURES AND REAGENTS

Primary monocytes. Blood was collected from healthy individuals through the UM Institutional Review Board Approved blood donor program (ID: HUM00024137. Date approved: 12/28/2010), which is in compliance with the Helsinki declaration. Each individual donated 20 ml of whole blood, and all of the samples were pooled for CD14+ cell isolation procedure. Blood (10–12 ml) was added to 25 ml of MACS buffer (Miltenyi Biotec; cat# 130-091-222), and then monolayers were separated by density gradient centrifugation with Ficoll-Paque™ PLUS (GE Healthcare; cat# 17-1440-02). This was followed by paramagnetic bead separation with CD14 microbeads (Miltenyi Biotec; cat# 130-050-201). Cells were plated in six-well cell culture plates (BD Falcon™; cat# 353046) in Gluta-MAX™-I RPMI Medium 1640 (Invitrogen; cat# 61870-036). If cytokines were added, they were added 1 h following the isolation step. IL4 (100 ng/μl; Humanzyme; Cat# HZ-1052) or 50 ng/μl MCSF (R&D Systems; Cat# 216-MC-005) was used to generate in-vitro M2 macrophage models. For in-vitro model of M1 macrophages, 100 ng/μl of IFNg (R&D Systems; Cat# 285-IF/CF) was added instead.

Cell lines. Human histomonocytic cell line U937 and human acute monocytic leukemia cell line THP-1 were obtained from the American Type Culture Collection. Cells were propagated using standard cell culture techniques and maintained in Gluta-MAX™-I RPMI Medium 1640 (Invitrogen; cat# 61870-036).

PREPARATION OF DYE–CONJUGATED miRNA PRECURSOR MOLECULES

The pre-miR™ hsa-miR-511 miRNA precursor molecules (Applied Biosystems; cat# PM10237), hsa-miR-1 miRNA precursor molecules (Applied Biosystems; cat# PM10617) and the scramble equivalent, Negative Control #1 (Applied Biosystems; cat# AM17110) were obtained from Applied Biosystems. The reverse transfection approach was chosen because the process would take 1 day less than traditional approaches and previous success had been reported [He et al., 2009].

Silencer siRNA-labeling kit (Applied Biosystems; cat# AM1632) was used to label the miRNA precursor molecules with Cy-3 dye to allow detection by fluorescence-activated cytometry. The conjugation was performed according to the manual, included the preparation of incubation of the labeling reagents with the precursor molecules at 37°C for 1 h. The dye-conjugated precursor molecules were then washed and dissolved in RNAse-free water to a final concentration of 25 μM.

PREPARATION OF miRNA–CONSTRUCT CONTAINING PLASMIDS

Plasmids used for transfections include the pEZX-MR04 nonlenti-vector plasmids that contained hsa-miR-511 precursor construct (Genecopoeia, cat# HmiR0155-MR04) and its scrambled control equivalent (Genecopoeia, cat# CmiR0001-MR04). The plasmids were propagated in One Shot TOP 10 E. Coli (Invitrogen; cat# C4040-10) and purified using QIAGEN Plasmid Maxi Kit (Qiagen; cat# 12162). After the purification steps, the plasmids were resuspended in TE buffer provided in the kit.

CHEMICAL TRANSFECTION WITH siPORT™ NeoFX™

siPORT™ NeoFX™(Applied Biosystems; cat# AM4511), a lipid-based transfection agent was used in the reverse chemical transfection performed according to the manufacturer's protocol. For transfection of each condition, 27 μl of NeoFX™, and 12 μl of either 25 μM of dye-conjugated miRNA precursors or 7 μg of pEZX-MR4 plasmids were added to 561 μl of OptiMem media (Invitrogen; cat# 11058-021), and incubated according to the manufacturer's protocol. The amounts of reagents used were based on optimization experiments performed beforehand. Finally, the incubated mix was transferred on a six-well tissue culture plate. Primary monocytes were layered on top of the transfection mixture to achieve a density of 1.5E06 cells/ml.

ELECTROPORATION WITH NUCLEOFECTOR™ II DEVICE

Electroporation was performed with the Human Monocyte Nucleofector Kit (Lonza; cat# VPA-1007) with the Nucleofector™ II Device (Lonza). Freshly isolated primary monocytes were resuspended in 100 μl of supplemented nucleofection solution and transferred to the provided cuvette. Either 12 μl of 25 μM of dye-conjugated miRNA precursors or 7 μg of pEZX-MR4 plasmids were mixed with the cell suspension. Subsequently, the cuvettes containing the cell suspension were nucleofected with Nucleofector™ II Device (Lonza) using recommended settings according to manufacturer's protocol. After electroporation, cells were quickly "recovered" by adding 500 μl serum-free RPMI media into the cuvette and then plated onto a six-well cell culture plate. The appropriate amount of serum-free RPMI media was added to achieve a cell density of 1.5e6 cells/ml.

FLUORESCENCE ACTIVATED CELL SORTING

Sample preparation. At 48 h post-transfection, primary monocytes were washed twice with sterile PBS and harvested by gently scraping the bottom of the wells with a cell scraper. They were then resuspended in sterile PBS supplemented with 0.2% BSA (Miltenyi Biotec). Incubation with CD206-APC dye-conjugated antibodies (Miltenyi Biotec; cat#130-095-217) was done at 1:10 dilution and 4°C for 40 min. To assess viability and exclusion of nonviable cells, a nucleic acid dye, 7-AAD (BD Pharminogen; cat# 559925) was added 10 min prior to analysis.

Cytometric analyses or FACS were done on FACSAria II cell sorter (BD Biosciences). Results were re-analyzed using FlowJo (Tree Star). The transfection efficiency was represented by the percentage of fluorescence positive cells.

REAL–TIME qPCR ANALYSIS

Total RNA was purified using Arcturus PicoPure RNA Isolation Kit (Applied Biosystems; cat# KIT0204) according to the manufacturer's protocol. DNase I was used to treat each sample during preparation step to prevent any DNA contamination of the RNA filtrate. Final RNA was collected in 11 μl of RNAse free H₂O. To assess the quality of the RNA, samples were submitted to the University of Michigan Sequencing Core where quality analysis was done with both NanoDrop spectrometer (Thermo Scientific) and Agilent 2100 Bioanalyzer (Agilent Bioanalyzer). Samples with an RNA integrity number (RIN) score of <7 were discarded. Taqman primers and

probe set for *PTK9* gene was purchased from Applied biosystems (cat# Hs00702289_s1). The *GAPDH* (glyceraldehyde 3-phosphate dehydrogenase) gene was used as a reference gene, to which *PTK9* expression levels were normalized. The taqman primers and probe set for *GAPDH* was also purchased from Applied biosystems (cat# Hs02758991_g1). This gene has been used as a reference gene for qRT-PCR analysis of primary human monocytes in literature [Puig-Kroger et al., 2009; Martinez-Nunez et al., 2011]. RT-qPCR plates were prepared as according to manufacturer's protocol, using High Capacity cDNA Reverse Transcription Kit (Applied Biosystems, cat# 4368814), and were analyzed on ABI HT7900 PCR system (Applied Biosystems). The relative mRNA expression level of *PTK9* in each sample was calculated using the comparative expression level $2^{-\Delta\Delta Ct}$ method.

To demonstrate biological activity of transfected miRNA precursors, pre-miR™ hsa-miR-1 miRNA precursor molecules (Applied Biosystems; cat# AM17150) was chemically transfected into the cells. At 48 h after transfection, successfully transfected cells were isolated with fluorescence-activated cell sorting. Immediately after isolation, cells were purified for RNA. Hsa-miR-1 was known to cause a miRNA-mediated degradation of *PTK9* (twinfilin-1) [Lim et al., 2005]. By comparing the expression levels of *PTK9* messengerRNA of miR-1 transfected cells compared to scrambled control transfected cells, the biological activity of miRNA precursor molecules could be validated.

LENTIVIRAL TRANSDUCTION

To demonstrate chemical transfection as the most suited method, it was compared to other common methods of transfection. GFP expressing lentiviral vector plasmid pMIRNA1 containing a miR-511-1 precursor construct (System Biosciences; Cat# PMIRH5111PA-1) and its corresponding scrambled control vector (System Biosciences; Cat# CD511B-1) were purchased from System Biosciences. The plasmids were packaged into lentiviruses by the University of Michigan Vector Core. Transduction of freshly isolated primary monocytes, U937 cells and THP-1 cells were performed at 1× viral titer. Primary monocytes were plated at a 1.5e6 cells/ml density, while the cell lines were plated at a 2e5 cells/ml density. Transduction of primary monocytes was also attempted at 5× viral titer, and with the addition of polybrene at 6.5 µg/ml.

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