Published in final edited form as:

Biotechnol Bioeng. 2015 August ; 112(8): 1632–1643. doi:10.1002/bit.25567.

MiRNA mimic screen for improved functional expression of neurotensin receptor

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Abstract

Obtaining adequate quantities of functional mammalian membrane proteins has been a bottleneck in their structural and functional studies because the expression of these proteins from mammalian cells is relatively low. To explore the possibility of enhancing expression of these proteins using miRNA, a stable T-REx-293 cell line expressing the neurotensin receptor type 1 (NTSR1), a hard-to-express G protein-coupled receptor (GPCR), was constructed. The cell line was then subjected to human miRNA mimic library screening. In parallel, an HEK293 cell line expressing luciferase was also screened with the same human miRNA mimic library. Five microRNA mimics: hsa-miR-22-5p, hsa-miR-18a-5p, hsa-miR-22-3p, hsa-miR-429 and hsa-miR-2110 were identified from both screens. They led to 48% increase in the expression of functional NTSR1 and to 239% increase of luciferase expression. These miRNAs were also effective in enhancing the expression of secreted glypican-3 hFc-fusion protein from HEK293 cells. The results indicate that these molecules may have a wide role in enhancing the production of proteins with biomedical interest.

Keywords

microRNA screen; neurotensin receptor; HEK293; protein expression

Introduction

Improving the expression level of recombinant mammalian proteins has not only been pursued by biotechnologist for production of commercial biotherapeutics, but has also been at the heart of numerous biomedical studies in academia, as an adequate supply of correctly folded proteins is a prerequisite for all structure and function studies. A critical area is

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mammalian integral membrane proteins such as receptors, ion channels and transporters which are encoded by 20–40% of all Open Reading Frames (ORFs) in the mammalian genome (Wallin and von Heijne 1998) and are targets of most of the medicines sold worldwide (Pierce et al. 2002). Even though more than 100,000 structures have been deposited in Protein Data bank, the overexpression of membrane protein remains difficult (Bill et al. 2011; Tate 2001) and only 898 membrane protein structures are available as of October 2nd, 2014 (<http://blanco.biomol.uci.edu/mpstruc/>). Rational attempts to improve membrane protein expression may not lead to expected results (Xiao et al. 2014) as membrane proteins involve particularly complex folding, assembly, and processing pathways, and there is only limited information for the bottlenecks that may reside in the protein production steps, such as transcription, translation, protein folding, secretion and cell viability.

MiRNAs have emerged as powerful tools for engineering cells with desirable properties (Jadhav et al. 2013), such as improved protein production capabilities (Fischer et al. 2014; Jadhav et al. 2014; Loh et al. 2014) and enhanced anti-apoptotic properties under stress conditions (Druz et al. 2013a; Druz et al. 2013b). MiRNAs are a novel class of small, non-coding RNAs that can simultaneously silence multiple genes by binding to their 3'-untranslated regions (3'-UTR) (Filipowicz et al. 2008). They exhibit a broad spectrum of regulatory effects in eukaryotic cellular processes including cell growth and apoptosis, cell differentiation and metabolism, cancer development and progression (Jadhav et al. 2013). Their capacity to globally regulate entire gene networks (Hobert 2008) and not introduce an additional translational burden (compared to gene overexpression strategies) (Hackl et al. 2012) makes them particularly advantageous for cell line development.

Here we present a high-throughput screening strategy for identifying miRNAs that can improve functional expression of the model membrane protein – Neurotensin Receptor type 1 (NTSR1) (Tanaka et al. 1990). Belonging to the G protein-coupled receptors (GPCRs) superfamily and interacting with its ligand neurotensin (Tyler-McMahon et al. 2000), NTSR1 plays important roles in Parkinson's disease, pathogenesis of schizophrenia, modulation of dopamine neurotransmission, hypothermia, and antinociception and in promoting growth of cancer cells (Bissette et al. 1976; Carraway and Plona 2006; Griebel and Holsboer 2012; Kitabgi 2002; Schimpff et al. 2001). The structure of a stabilized NTSR1 mutant with T4 lysozyme replacing most of the third intracellular loop was recently determined (White et al. 2012). Previously, we constructed inducible suspension mammalian HEK293 cells expressing functional NTSR1, that allowed us to obtain 1mg purified receptor per liter of cell culture with a viable cell density of 1.4 million cells/mL (Xiao et al. 2013). Here we explore the ability to improve receptor expression by applying the powerful miRNA tool. In this manuscript we describe the implementation of high-throughput image-based screen with NTSR1-GFP-expressing cells using a human miRNA mimic library comprising 875 miRNA mimics.

Materials and methods

Construction of expression plasmid pJMA-NTSR1-GFP

Truncated wild type NTSR1 (T43-K396) was subcloned into the tetracycline inducible plasmid pJMA111 (a kind gift from Dr. Christopher G. Tate) replacing the serotonin transporter construct using KpnI and NotI restriction sites. Thus NTSR1 was placed downstream of the tetracycline-controlled CMV promoter and had an eGFP-deca-histidine tag fused to its C-terminal (Fig. S1) (Abdul-Hussein et al. 2013).

Construction of stable NTSR1-GFP-expressing T-REx-293 cell line

The T-REx-293 cell line was maintained as an adherent culture in DMEM containing 10% certified FBS and 5 µg/mL blasticidin (Invitrogen). The cells were transfected with the plasmid pJMA-NTSR1-GFP using Lipofectamine 2000 according to the manufacturer's protocol (Life Technologies). One day after transfection, cells were transferred into fresh DMEM medium containing 200 µg/mL zeocin (Invitrogen) and the medium was replaced every three days. Two weeks later, ten cell clones were separately expanded into two T-flasks each. Cells in one T-flask were harvested during the exponential growth phase and frozen in 10% DMSO for storage. Cells in the other T-flask were induced with 1 µg/mL tetracycline for 24 hrs, after reaching 80% confluency. Cells were then detached from the flask and washed with cold PBS. After adjusting the cell density to $\sim 1 \times 10^6$ cells per mL, protease inhibitors (Roche) were added and the cell suspension was frozen on dry ice in 1 mL aliquots. NTSR1 expression levels were determined by [³H]NT binding and the clone with the highest expression level was selected for further experiments. The selected stable T-REx-293-NTSR1-GFP high expressor was then routinely maintained in DMEM containing 10% certified FBS, 5 µg/mL blasticidin and 200 µg/mL zeocin.

High-throughput miRNA screen

T-REx-293-NTSR1-GFP cells were screened with a miRNA mimic library (Qiagen) based on Sanger miRBase 13.0 and consisting of 875 miRNAs mimics. For transfection, 0.8 pmol of each mimic was spotted to 384 well plate wells (Corning) and 20 µL of serum-free DMEM containing 0.1 µL of Lipofectamine RNAiMax (Life Technologies) was then added to each well. This lipid-miRNA mixture was incubated at ambient temperature for 30 min prior to adding 2000 cells in 20 µL of DMEM containing 20% certified FBS (Gibco). Transfected cells were incubated at 37°C in 5% CO₂ for 72 hours and induced with 1 µg/mL tetracycline for 24 hours for NTSR1-GFP expression. Cells were then fixed with 2% paraformaldehyde (Electron Microscopy Sciences), stained with Hoechst 33342 (Life Technologies) for 45 minutes and gently washed with PBS. Plates were imaged with an ImageXpress Micro XL (Molecular Devices). Total cell number and per cell green fluorescence intensity were calculated using MetaXpress software (Molecular Devices) employing the Multi-Wavelength Cell Scoring application module. All screening plates had a full column (16 wells) of Silencer Select Negative Control #2 (Life Technologies) and the median value of each plate's negative control column was used to normalize corresponding sample wells. A full column of positive control siRNA targeting GFP (GFP-22 siRNA, Qiagen) was also used as on-plate reference for transfection efficiency. The median absolute deviation (MAD) – based z-score was calculated for each sample (Chung et al. 2008).

Validation transfection

Validation transfections were performed in 12-well plates with miScript miRNA mimics (Qiagen, Cat.No. 219600-S0), SilencerSelect Negative Control #2 and lethal control siRNA (Qiagen AllStars Cell Death Control) served as a control for transfection efficiency. Cells were transfected as described for screening except 0.15 million cells were transfected with 40nM miRNA using 6.25ul Lipofectamine RNAiMax in a total volume of 1mL of media. 72 hours after transfection, cells were induced with 1µg/mL tetracycline. 24 hours later, cells from each well were detached with non-enzymatic cell dissociation buffer (Gibco, Cat. No. 13150-016) and washed twice with cold PBS. Cell densities and viability were determined by trypan blue exclusion using a CEDEX cell quantification system (Roche, Mannheim, Germany). Based on the counts, cell densities were adjusted to 0.5 million cells/ml with PBS and then subject to flow cytometry analysis. The remaining cells were pelleted and frozen on dry ice for [³H]NT binding assays.

Flow cytometry analysis

Cells harvested from validation transfection step were diluted to 0.2 million cells/ml with cold PBS for flow cytometry analysis. Green fluorescence was measured with Guava EasyCyte 5HT and InCyte software (Millipore). The green fluorescence signal and cell gating were adjusted using uninduced T-REx-293-NTSR1-GFP cells, with more than 99% of the cells in low fluorescence range (<100). The setting was kept same for acquisition of all cell samples.

Analytical solubilization of NTSR1

The detergents *n*-Dodecyl-β-D-maltoside (LM), 3-[(3-cholamidopropyl) dimethylammonio] -1- propane sulfonate (CHAPS) and cholesteryl hemisuccinate Tris salt (CHS) were obtained from Anatrace. Cell pellets from 2 mL of suspension culture were suspended in Tris-glycerol-NaCl buffer. Then the detergents LM, CHAPS and CHS were added to give a final buffer composition of 50 mM TrisHCl pH 7.4, 200 mM NaCl, 30% (v/v) glycerol, 1% (w/v) LM, and 0.6% (w/v) CHAPS and 0.12% (w/v) CHS in a total volume of 0.5 mL. The samples were placed on a rotating mixer at 4°C for 1 hour. Cell debris and non-solubilized material were removed by ultracentrifugation (TL100 rotor, 60k rpm, 4°C, 30 min in Optima Max bench-top ultracentrifuge, Beckman), and the supernatants containing detergent-solubilized NTSR1 were used to determine the total number of expressed receptors by a detergent-based radio-ligand binding assay (see below).

Ligand binding assay

Tritiated neurotensin agonist [³H]NT ([3,11-tyrosyl-3,5-³H(N)]-pyroGlu-Leu-Tyr-Glu-Asn-Lys-Pro-Arg-Arg-Pro-Tyr-Ile-Leu) was purchased from Perkin Elmer. Ligand-binding assays with detergent-solubilized receptors were carried out in TEBB assay buffer (50mM Tris pH 7.4, 1mM EDTA, 40µg/mL bacitracin, 0.1% BSA) containing 0.1% (w/v) LM, 0.2% (w/v) CHAPS and 0.04% (w/v) CHS. For one-point assays, receptors were incubated with 2 nM [³H]NT on ice for 1 hour in a volume of 150 µL. The concentration of [³H]NT used was at least 5-fold above the apparent dissociation constants for detergent-solubilized NTSR1 to allow high receptor occupancy. Separation of the receptor-ligand complex from free ligand

(100 μ L) was achieved by centrifugation-assisted gel filtration using Bio-Spin 30 Tris columns (BioRad), equilibrated with RDB buffer (50 mM Tris-HCl, pH 7.4, 1 mM EDTA, 0.1% (w/v) LM, 0.2% (w/v) CHAPS, 0.04% (w/v) CHS). Non-specific [3 H]NT binding of 220 dpm was subtracted from total binding to calculate the total amount of receptors in T-REx-293-NTSR1-GFP cells. The number of functional NTSR1 was estimated by specific [3 H]NT binding assuming one ligand-binding site per receptor molecule. The number of cells in the assay was derived by cell counting at cell harvest. This approach led to the calculation of the parameter “receptors/cell”.

Validation with luciferase-expressing cells

CMV-Luc2-HygroHEK293 cell line constitutively expressing luciferase is purchased from Promega. Validation transfections were performed in 12-well plates with miScript miRNA mimics (Qiagen, Cat.No. 219600-S0), SilencerSelect Negative Control #2 and lethal control siRNA (Qiagen AllStars Cell Death Control) served as a control for transfection efficiency. Cells were transfected in duplicates as described above in the screening method with the following modification: 0.1 million cells were transfected with 40nM miRNA using 6.25 μ L Lipofectamine RNAiMax in a total volume of 1 mL of media. 72 hours after transfection, 500 μ L of ONE-GloTM Reagent (Promega) was added to one set of replicates for luciferase activity quantification and 500 μ L of CellTiter-GloTM Reagent (Promega) was added to the second set of replicates for viable cell density measurement. All plates were incubated at room temperature for 20 minutes to stabilize luminescent signal and then measured with SpectraMax i3 plate reader (Molecular Devices). Per cell luciferase production was calculated from overall luciferase activity and viable cell number.

Validation with GPC3-hFc-expressing cells

HEK- GPC3-hFc cell line constitutively secreting glypican-3 hFc-fusion protein (GPC3-hFc) is a kind gift from Dr. Mitchell Ho's group from the National Cancer Institute of the NIH. Cells were grown in DMEM supplemented with 10% FBS in a humidified incubator set at 37°C and 5% CO₂.

Cells were transfected in 12-well plates as described above in the screening method with the following modification: 0.15 million cells were transfected with 40nM miRNA using 6.25 μ L Lipofectamine RNAiMax in a total volume of 1 mL of media. 6 days after transfection, cell culture supernatant was collected and cleared using centrifuge for GPC3-hFc concentration determination with ELISA and cells were detached and counted by trypan blue exclusion using a CEDEX cell quantification system (Roche, Mannheim, Germany). Per cell GPC3-hFc production can be calculated from overall GPC3-hFc yield and viable cell number.

ELISA for GPC3-hFc concentration determination

AffiniPure F(ab')₂ Fragment Goat Anti-Human IgG (min X Bov, Ms, Rb Sr Prot, Cat. 109-006-170, Jackson Immunology) was used to coat a 96-well plate at 5 μ g/mL in PBS buffer, 50 μ L per well, at 4 °C overnight. After the plate was blocked with 2% BSA in PBS buffer, pre-diluted cell culture supernatant was added, and the plate was incubated at room temperature for one hour to allow binding to occur. After the plate was washed twice with PBS containing 0.05% Tween 20, Peroxidase-conjugated AffiniPure Goat-anti-uman IgG

(Cat. 109-035-098, Jackson Immunology) was added at 1:4000 dilutions, 50ul/well. Following incubating at room temperature for one hour, the plate was washed 4 times and detected with Peroxidase Substrate System (KPL).

Results

Construction of inducible T-REx-293-NTSR1-GFP cell line for image-based screen

A stable cell line expressing functional wild type NTSR1-GFP fusion was constructed using the inducible T-REx system(Yao et al. 1998) by transfecting T-REx-293 cells with the pJMA-NTSR1-GFP plasmid (Fig. S1). Ten clones were isolated and their neurotensin receptor expression level upon tetracycline induction was measured by [³H]NT binding assay (data not shown). A high-expressing clone producing 8.4 million receptor molecules per cell was selected for further experiments. As seen in Fig. 1, the receptors for this clone are located mostly on the plasma membrane as expected.

High-throughput miRNA screen for enhanced NTSR1-GFP expression

To identify miRNAs that improve NTSR1 expression in T-REx-293-NTSR1-GFP cells, the cells were screened with a library comprised of 875 human miRNA mimics. Cells were transiently transfected with mimics in 384-well format for 72 hours followed by tetracycline-induced expression of NTSR1-GFP fusion protein (Fig. 2A). Twenty four hours after induction, the cells were fixed followed by nuclear staining. Each well was then imaged to obtain total cell number and per cell mean green fluorescent intensity (Fig. 2B). Sample values were normalized based on the median value of each plate's negative control column. A column of positive control siRNA capable of silencing *gfp* gene was also used as on-plate control for transfection efficiency. GFP-directed siRNA consistently provided a > 80% decrease in green fluorescence intensity. To assess reproducibility, the screen was performed in duplicate, resulting in a correlation coefficient of 0.92 (Fig. 2C). Furthermore, the screen was completed again in replicate using cells from a different passage. The correlation between the two independent screens was 0.73. The median absolute deviation (MAD) – based z-score(Chung et al. 2008) was calculated for each sample, and the distribution of miRNA activity is plotted in Fig. 2D. 40 miRNAs were shown to significantly increase NTSR1-GFP productivity (by passing the 2.0 MAD thresholds. Table 1) in both biological replicates and 26 of them (two thirds of total 40) were selected for follow up analysis. All screen data for the four replicates can be found in Table S1.

Validation of the selected miRNA candidates by flow cytometry analysis

The expression level of NTSR1-GFP following transient transfection of the cells with the top 26 microRNA was measured by flow cytometry (Fig. 3). The un-induced cells exhibited basal GFP expression with only 1% of cells exceeding the background fluorescence (10^1) (Fig. 3A). Following transfection with negative control siRNA (siN.C.) and tetracycline induction, the expression of NTSR1-GFP caused a significant shift in the fluorescence intensity, resulting in a geometric mean of fluorescence (MOF) of 138. A further shift was observed when the cells were transfected with various miRNA mimics followed by tetracycline induction, including miR-129-5p, which led to a MOF of 197. Compared with negative control siRNA, 14 of the 26 miRNAs resulted in an increased MOF. From this

group, top 9 miRNAs were selected for further investigation (Fig. 3B). Following the transfection with the 26 selected miRNAs, a large variance was seen in viable cell density (ranged from 54% to 135%, normalized to negative control) but not in viability (ranged from 84% to 97%) (Fig. 3C).

[³H]NT binding assay validation for improved functional expression of NTSR1

The effect of the top 9 miRNAs on the functional expression of NTSR1 was also evaluated by measuring the functional activity of the receptor through the binding of labeled neurotensin ([³H]NT). Although all top 9 miRNAs were shown to improve NTSR1-GFP expression based on GFP fluorescence, only 5 of them (miR-22-5p, miR-18a-5p, miR-22-3p, miR-429 and miR-2110) led to improved functional activity levels of NTSR1 (Fig. 4A). Of these, miR-2110-transfected cells expressed 13.8 million functional neurotensin receptor molecules per cell, which was 48% higher than that from siN.C. In addition, miR-22-5p and miR-22-3p improved functional expression of NTSR1, by 30% and 21% respectively. As seen in Fig. 4B a number of the top 9 miRNAs had negative effect on cell growth and viability.

MiRNA screen for enhanced luciferase expression

The human mimic miRNA library was also evaluated for its effects on the expression of luciferase in HEK293 cells constitutively expressing luciferase under control of a cytomegalovirus (CMV) promoter. Screening was performed in duplicate in 384-well format. Seventy two hours post- transfection, one set of plates was assayed for luciferase and the other set was used for viable cell density (Fig. 5A). Both luciferase activity and viable cell density were normalized to the median value of each plate's negative control column and the luciferase expression per cell was calculated for each miRNA. Though luciferase and NTSR1 screen exhibited a limited correlation ($R=0.31$, Fig. 5B), seven out of nine top hits identified from NTSR1 screen (Fig. 5C) also significantly improved per cell luciferase productivity on a per cell basis (passing the >2.0 MAD threshold).

Validation of common hits

The top 9 miRNAs identified from the NTSR1 screen were examined for their effects on luciferase activity in a 12-well plate format where seven miRNAs improved luciferase activity. (Fig. 6A). MiR-892b and miR-22-3p showed the largest effect on luciferase expression with a 239% and 207% improvement respectively. Although these microRNAs inhibited cell growth (Fig. 6B), the overall production of luciferase from cells transfected with miR-892b and miR-22-3p was still 188% and 127% higher, respectively, than the negative control siN.C. level (Fig. 6C). Interestingly, both miR-22-3p and miR-22-5p showed up as top common hits for NTSR1 and luciferase screen.

Application of top common hits on secreted protein

To investigate the impact of top common hits on secreted protein production, the five identified miRNAs (hsa-miR-22-5p, hsa-miR-18a-5p, hsa-miR-22-3p, hsa-miR-429 and hsa-miR-2110) were independently transfected into HEK293 cell line stably expressing secreted hFc-fusion protein: glypican-3 hFc-fusion protein (GPC3-hFc) (Feng et al. 2013). All five

miRNAs enhanced per cell GPC3-hFc secretion (up to 120% improvement, Fig. 7A), while three miRNAs (hsa-miR-22-5p, hsa-miR-18a-5p and hsa-miR-22-3p) effectively enhanced overall GPC3-hFc (up to 62%, Fig. 7C).

Discussion

Integral membrane proteins such as mammalian receptors, ion channels and transporters are vital for medical research. However, obtaining large amounts of functional membrane proteins for medical research, especially structural studies, has been difficult and therefore been a barrier for productive research towards better understanding of their mechanisms and potential medical use (Andrell and Tate 2013; Bill et al. 2011; Tate 2001). So far, a tetracycline-inducible mammalian expression system (Yao et al. 1998) has been shown to be an effective method for functional expression of membrane proteins (Dostalova et al. 2010; Reeves et al. 2002; Tate et al. 2003). This inducible system together with optimized production conditions led to a yield of 1 milligram per liter of purified NTSR1 (Xiao et al. 2013). Compared with well-developed prokaryotic hosts such as *E. coli*, the production of membrane proteins from higher eukaryotic hosts is still in the stage of “trial and error” (Wagner et al. 2006) since engineering tools are limited and the membrane protein synthesis, insertion, folding and trafficking are not completely understood (Xiao et al. 2014).

To improve the production of these proteins, a bottom-up screening approach using human miRNA mimics library was implemented to identify candidates that lead to improved expression of the GPCR from the T-Rex-293 cells. This approach has previously proven effective for apoptosis screening (Druz et al. 2013a) and recombinant secreted protein screening (Fischer et al. 2014; Strotbek et al. 2013) in CHO cells. In this study, we developed an image-based high-throughput screening method to detect per cell green fluorescence signal, which is applied as a proxy for the number of molecules of NTSR1 protein expressed per cell. In addition to its high reproducibility (0.92 correlation between technical replicates), this method is cost-effective for protein with a fluorescent label, as no secondary reagent is needed for protein quantification. It is also high throughput and high-capacity, as cells are fixed and the screening is not time-sensitive compared to live-cell processes such as flow cytometry. This screen methodology can be applied to other membrane or intracellular protein candidates when the targeted protein is fused with GFP. Although GFP fusion has been widely used for membrane protein overexpression screen and purification in a variety of hosts (Drew et al. 2006; Newstead et al. 2007; Thomas and Tate 2014), it is possible that the N-terminal GFP fusion may mask signal sequence essential for protein insertion. This may compromise folding or correct localization of the desired membrane protein (Palmer and Freeman 2004; Wiemann et al. 2001). C-terminal GFP fusion, on the other hand, is preferable as it is generally better in maintaining the localization and function of the native protein (Palmer and Freeman 2004) with exceptions when C-terminal contains an essential functional segment (Huh et al. 2003; Sato et al. 2001).

Among the 875 human miRNA mimics tested, 40 mimics consistently led to significant improvement in per cell green fluorescence levels, exhibiting an average MAD-based-z-score higher than 2.0. Among the top 40 candidates, miR-892b, miR193b-3p and miR-193a-3p share the same seed sequence (ACUGGC), indicating that they may comprise

an overlap in target genes. Similarly, miR-129-3p and miR-129-1-3p also share a same seed sequence (AGCCCU).

The activity of two thirds of the 40 mimics was confirmed further by flow cytometry and the 9 mimic candidates contributing to the highest per cell green fluorescence signal were further tested in the [³H]NT binding assay. Five out of the nine mimics showed up to 48% improvement in functional expression of NTSR1. From these five, miR-2110 is a novel miRNA that has been identified but not studied (Zhu et al. 2009). The other four miRNAs (miR-429, miR-18a-5p, miR-22-5p and miR-22-3p) have been associated with cancer research in which they have exhibited contradicting effects on cell proliferation, cell growth, and protein production depending on the cell type and stage of cell development (Jadhav et al. 2014; Lang et al. 2014; Liu et al. 2014; Song et al. 2013; Song et al. 2014; Takakura et al. 2008; Xiong et al. 2010). For example, miR-429, a member of the miR-200 family, was shown to suppress tumor growth in human osteosarcoma (Liu et al. 2014), while in non-small cell lung cancer (NSCLC), the same miRNA is suggested as a potential target for NSCLC due to its promotion of cell proliferation (Lang et al. 2014). MiR-18a-5p is part of the miR-17-92 precursor sequence cluster, which is also named Oncomir-1. This miR-17-92 cluster was studied in depth regarding its effect on recombinant EpoFc protein production in CHO cells. Although over-expression of the entire cluster decreased productivity while having no effect on cell growth, the over-expression of miR-17 and miR-92 were shown to increase production (Jadhav et al. 2014).

Of the nine miRNAs that enhanced the expression of the NTSR1-GFP fusion protein, four (miR-129-5p, miR-221-5p, miR-892b and miR-639) were not associated with enhanced binding activity of the agonist in the [³H]NT assay. This may be an indication that NTSR1 could be misfolded in these cells following the enhanced expression. Another observation is that eight of the nine top hits (except miR-129-5p) caused a decrease in the viable cell number. One possible reason for this behavior is that overexpression of NTSR1-GFP could be toxic to the cells. Another possibility is that the introduction of a specific miRNA to the cells is associated with a growth arrest, leading to improved protein production (Barron et al. 2011; Jadhav et al. 2013). Since multiple pathways and genes can be targeted by one miRNA, it will be worthwhile to examine which specific genes are down-regulated in these cells and to investigate the mechanism that improved NTSR1 functional expression.

In parallel to the analysis of the miRNA effect on the NTSR1 expression, an HEK293 cell line constitutively expressing luciferase under the control of CMV promoter was subjected to a screening of the same miRNA mimics library. This screen showed low degrees of correlation ($R = 0.31$) with the NTSR1-GFP screen. The low correlation may be the result of the difference between biogenesis process of integral membrane proteins and intracellular soluble proteins; the difference between constitutive expression elements and the inducible expression system; and clonal differences between the two HEK293 cell line used. Despite the overall low correlation between the screens, seven out of nine top miRNAs (except miR-129-5p and miR-639) identified from NTSR1-GFP screen, improved luciferase activity from 50% to 239%. All the final five miRNAs (miR-429, miR-18a-5p, miR-22-5p and miR-22-3p and miR-2110) capable of improving NTSR1 functional expression were also relevant for improving luciferase expression.

These five miRNAs affecting both model proteins were expected to have wider application for other types of proteins. Therefore, they were tested with HEK293 cell line constitutively secreting a Fc fusion proteins with medical importance (Feng et al. 2013). All of the five miRNAs were effective in enhancing per cell Fc fusion protein secretion. However, the overall Fc fusion protein yield varied from 10% decrease to 62% increase, partially depending on the viable cell number after miRNA transfection.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

This work was supported by the Intramural Research Program of the National Institutes of Health (SX, JS: National Institute of Diabetes and Digestive and Kidney Diseases; YC, SM: National Center for Advancing Translational Sciences) and was partially supported by NIH grant 5R01GM095685-03. DNA sequence synthesis and analysis were performed at the Center for Biologics Evaluation and Research (Food and Drug Administration, Bethesda, MD, USA). We thank Sarah Inwood (National Institutes of Health, Bethesda, USA) for discussion and help with miRNA references. We are grateful to Dr. Reinhard Grishammer and Jim White (National Institutes of Health, Rockville, US) for providing NTSR1 construct, ligand binding assay workstation and the kind help. We thank Dr. Christopher G. Tate (MRC Laboratory of Molecular Biology, Cambridge, UK) for the kind gift of pJMA111 plasmid and Dr. Mitchell Ho (National Cancer Institute, Bethesda, US) for HEK-GPC3-hFc cell line.

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Fig. 1.
Confocal microscopy of tetracycline-induced T-REx-293-NTSR1-GFP cells with NTSR1-GFP fusion protein located on plasma membrane.

Fig. 2. miRNA screen with stable T-REx-293-NTSR1-GFP cell line. (A) Workflow of the screen. 72 hours post-transfection with human mimic miRNA library (875 miRNAs) in 384-well format, cells were induced with tetracycline, with fixation and analysis 24 hours later. (B) Nuclei staining and GFP expression was captured by an ImageXpress Micro XLSs. (C) Correlation plot of replicates from the miRNA library screen. The correlation coefficient is 0.92. (D) Distribution of miRNA mimics activity on improved NTSR1 expression; top hits (passing 2.0 MAD thresholds) are highlighted.

Fig. 3.

Flow cytometry analysis on T-REx-293-NTSR1-GFP cells transfected with 26 miRNAs selected from those scoring > 2 MAD. (A) Fluorescence histogram of uninduced cells (grey), induced cells transfected with negative control siRNA siN.C. (dash line) and induced cells transfected with miR-129-5p (solid line). Transient transfection of miR-129-5p caused an increase in fluorescence intensity as shown by a right shift compared to control. (B) Testing of the 26 miRNA screen hits by flow cytometry analysis. Cells were transiently transfected with the indicated miRNAs in 12-well plate format and induced with

tetracycline. MOF from each sample was normalized to the negative control (siN.C.). Three biological samples were collected for each transfection experiment. Top 9 miRNAs are indicated. (C) Normalized viable cell density and viability of cells transfected with 26 miRNA hits. Error bars represent SEM (standard error of the mean).

Fig. 4.

Validation of improved functional expression of NTSR1 with [^3H]NT binding assay. (A) Functional NTSR1 numbers were determined by [^3H]NT binding assays using detergent solubilized cells. (B) Cells were counted at harvest and normalized to the control (siN.C.). Two independent experiments were carried out with different passages of T-REx-293-NTSR1-GFP cells, and each independent experiment was tested in duplicate. Error bars indicate SEM.

Fig. 5. miRNA screen with stable HEK-CMV-Luc2-Hygro cell line. (A) Workflow of the screen. Transfection with the human miRNA mimic library was performed in duplicate in 384-well format. 72 hours post transfection, one replicate was used for luciferase measurement and the other one was subject to cell viability assay. Data from the two sets of plates were used to calculate per cell luciferase expression level. (B) Correlation plot of screen result from luciferase screen and NTSR1-GFP screen. (C) Top common hits from miRNA library screen with NTSR1 and luciferase as target protein.

Fig. 6.

Validation of improved luciferase activity. CMV-Luc2-Hygro cells were transfected in 12-well plates with the top 9 miRNAs in duplicate. 72 hours post transfection, one replicate was used for luciferase measurement and the other one was subject to cell counting. The experiment was performed twice with different passages of cells. (A) Per cell luciferase activity was determined by ONE-Glo luciferase assay and viable cell density. (B) Viable cell density and (C) Overall luciferase production were normalized to the negative control (siN.C.). For each biological sample, the measurement was done in duplicates. Error bars indicate SEM.

Fig. 7.

Improved glypican-3(GPC3) hFc-fusion protein secretion by the five top miRNAs. (A) Per cell GPC3-hFc secretion was determined by ELISA and viable cell density. (B) Viable cell density. (C) Overall GPC3-hFc production was normalized to the negative control (siN.C.) The experiment was performed twice with different passages of cells. For each biological sample, the measurement was done in triplicates. Error bars indicate SEM.

Table 1

Top hits from human miRNA mimics screen based on per cell green fluorescence intensity (MAD>2.0)

Human miR ID (hsa-)	Variant	Mature miRNA sequence	MAD-based z-score	Signal relative to negative control (%)
miR-221	5p	ACCUGGCAUACAAUGUAGAUUU	5.3	248
miR-429	–	UAAUACUGUCUGGUAAAACCGU	4.2	212
miR-22	5p	AGUUCUUCAGUGGCAAGCUUUA	4.0	215
miR-892b	–	CACUGGCUCCUUUCUGGGUAGA	3.7	201
miR-1974	–	UGGUUGUAGUCCGUGCGAGAAUA	3.6	201
miR-210	3p	CUGUGCGUGUGACAGCGGCUGA	3.2	183
let-7f-2	3p	CUAUACAGUCUACUGUCUUUCC	3.0	178
miR-130b	5p	ACUCUUUCCUGUUGCACUAC	2.9	178
miR-188	5p	CAUCCCUUGCAUGGUGGAGGG	2.9	177
miR-301a	3p	CAGUGCAAUAGUAUUGUCAAAGC	2.9	176
miR-129	5p	CUUUUUGCGGUCUGGGCUUGC	2.7	172
miR-147a	–	GUGUGUGGAAAUGCUUCUGC	2.6	168
let-7c	5p	UGAGGUAGUAGGUUGUAUGGUU	2.6	168
miR-1909	5p	UGAGUGCCGGUGCCUGCCUG	2.6	169
miR-138-1	3p	GCUACUUCACAACACCAGGGCC	2.5	167
miR-193b	3p	AACUGGCCCUCAAAGUCCCGCU	2.5	166
miR-650	–	AGGAGGCAGCGCUCUCAGGAC	2.5	163
miR-639	–	AUCGCUGCGGUUGCGAGCGCUGU	2.4	165
miR-10b	3p	ACAGAUUCGAUUCUAGGGGAAU	2.4	162
miR-2110	–	UUGGGGAAACGGCCGCUGAGUG	2.3	160
miR-22	3p	AAGCUGCCAGUUGAAGAACUGU	2.3	158
miR-193a	3p	AACUGGCCUACAAAGUCCAGU	2.3	156
miR-340	3p	UCCGUCUCAGUACUUUAUAGC	2.3	159
miR-649	–	AAACCUGUGUUGUUAAGAGUC	2.0	150
miR-18a	5p	UAAGGUGCAUCUAGUGCAGAUAG	2.0	149
miR-192	3p	CUGCCAAUCCAAGGUCACAG	2.0	148

All mature miRNAs variants are shown: 5'-end pre-miRNA derived (marked 5p) and 3'-end pre-miRNA derived (marked 3p)