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Identifying HIPK1 as target of miR-22-3p enhancing recombinant protein production from HEK 293 cell by using microarray and HTP siRNA screen

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Abstract

Protein expression from human embryonic kidney cells (HEK 293) is an important tool for structural and clinical studies. It was previously shown that microRNAs (small, noncoding RNAs) are effective means for improved protein expression from these cells, and by conducting a high-throughput screening of the human microRNA library, several microRNAs were identified as potential candidates for improving expression. From these, miR-22-3p was chosen for further study since it increased the expression of luciferase, two membrane proteins and a secreted fusion protein with minimal effect on the cells' growth and viability. Since each microRNA can interact with several gene targets, it is of interest to identify the repressed genes for understanding and exploring the improved expression mechanism for further implementation. Here we describe a novel approach for identification of the target genes by integrating the differential gene expression analysis with information obtained from our previously-conducted high-throughput siRNA screening. The identified genes were validated as being involved in improving luciferase expression by using siRNA and qRT-PCR. Repressing the target gene, HIPK1, was found to increase luciferase and GPC3 expression 3.3-fold and 2.2-fold respectively.

Keywords

microRNA; siRNA; miR-22-3p; microarray; HIPK1; protein expression

1 Introduction

Enhancing recombinant protein expression from mammalian cells is of interest to the pharmaceutical and the biotechnology fields for the purpose of obtaining proteins needed for

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Conflict of Interest

The authors declare no financial or commercial conflict of interest.

therapeutic, biochemical and structural studies [1]. Current approaches for improving protein production are based on modifying growth strategies, optimizing media composition, genetically altering the producing cells, [2] and by improving the downstream processing [3]. Recently, the use of small non-protein-coding RNA, microRNA or siRNA, has been shown to be a promising methodology for improving protein expression by identifying specific genes and by altering their expression [4–7].

MicroRNAs, are natural cells products, approximately 22 nucleotides in length that, in their native form, modify gene-expression post-transcriptionally by competitive binding to mRNA repressing translation or causing mRNA destabilization [8]. The microRNA active site is approximately seven base pairs at the 5’ end of the molecule known as the “seed” which can target multiple genes. Single microRNA can, therefore, affect the expression of more than one gene and several bioinformatics algorithms are available to predict these genes [9]. The change in expression of multiple direct and indirect gene targets, can have a synergistic effect whereby small changes of multiple genes can have a significant effect on different pathways including recombinant protein production [5].

Expression of various recombinant proteins including secreted proteins, membrane proteins, antibodies, and viral vectors, has been found to be improved following the addition or deletion of microRNAs. The search for the specific affected genes was done by using microarrays, high-throughput screening, next generation sequencing, and/or quantitative reverse transcription PCR (qRT-PCR) [4, 6, 10]. While these studies did not describe the specific mechanism by which the microRNA acted, they identified genes and pathways involved in apoptosis, HDAC5 modulation, and the ubiquitin pathway [11–14].

Most studies associated with microRNA effect on protein production have been done in Chinese hamster ovary cells (CHO) which are the chosen producers for monoclonal antibodies that are being used for therapeutic purposes [4]. In this work, we concentrated on human embryonic kidney (HEK) cells that can perform post-translational modification and therefore are alternatives for expression of specific proteins such as human coagulation factors, growth factors and hormones [15, 16]. Also, compared with the CHO genome, the human genome is more widely understood and therefore more tools such as microarrays, microRNA mimics and siRNAs have been created.

In previously conducted high-throughput human microRNA screen in HEK 293 cells, we identified miR-22-3p as a promising candidate for improved expression of luciferase (Luc) reporter, two hard-to-express membrane proteins, Neurotensin Receptor type 1 (NTSR1) and Seretonin transporter (SERT), as well as secreted glypican-3 hFc-fusion protein (GPC3-hFc) [17]. Following this finding, we decided to identify genes affected by this specific microRNA through the implementation of differential gene expression analysis together with data obtained from our previously conducted genome scale siRNA study [7]. We believe that the results of this combined approach can provide better understanding of the mechanism by which this specific microRNA improved recombinant protein expression.

2 Materials and methods

2.1 Cell lines and cultures

A CMV-LUC2-HygroHEK293 cell line constitutively expressing firefly luciferase was purchased from Promega. An HEK 293 cell line constitutively expressing glypican-3 hFc-fusion protein (GPC3-hFc) was a gift from Dr. Mitchell Ho. Experiments were completed with cells between passage numbers 6 and 30. Cells were maintained in 10% FBS Dulbecco's Modified Eagle Medium (DMEM) in a humidified incubator set at 5% CO2 and 37°C.

2.2 Transient miRNA and siRNA transfection

Transfections were performed in 24-well plates with miScript miRNA mimics (Qiagen) or SilencerSelect siRNA (Life Technologies), a SilencerSelect Negative Control #2 or a lethal AllStars death control siRNA (Qiagen). Three different cultures (biological triplicates) of cells were transfected in duplicates. In each well, 250 μL of serum-free DMEM containing 3.25 μL Lipofectamine RNAiMax (Life Technologies) was added to 20 pmol of miRNA or siRNA. After incubation at room temperature for 15 min, 75,000 cells in 250 μL of 20% FBS supplemented DMEM were added. The plates were then incubated at 5% CO2 and 37°C.

2.3 Luciferase activity, western blot and cell viability assays

Luciferase expressing cells were transfected as above. Seventy-two hours after transfection, cells were transferred to 96-well plate for luciferase and cell viability assays. The remainder of the cells were concentrated into a pellet for RNA extraction. The cells in the 96-well plate were measured for luciferase with ONE-Glo™ Reagent (Promega) and for viability with CellTiter-Glo™ Reagent (Promega), using a SpectraMax i3 plate reader (Molecular Devices) according to the manufacturer’s protocol. Luciferase per cell was calculated by dividing the relative light units of the luciferase assay by the relative light units of the cell viability assay. Percentages were calculated dividing the result by that of the negative control and multiplying by 100.

For the western blot, luciferase expressing cells were transfected as above in duplicates. Seventy-two hours after transfection cells were washed with cold PBS and lysed using RIPA buffer with protease and phosphatase inhibitor cocktail (ThermoFisher Scientific). Lysates from the duplicate wells were combined and diluted with PBS to equal concentrations. Proteins were separated with a NuPAGE 4–12% bis-tris gel (ThermoFisher Scientific) at 200 V for 50 min and transferred to a nitrocellulose membrane using the iBlot Gel Transfer System (Invitrogen) using P8 for 8 min. This was then used for immunodetection with mouse anti luciferase at a 1:1,500 dilution (ThermoFisher Scientific) and mouse anti-β-actin at a 1:1,000 (Sigma) as primary antibodies and an HRP conjugated goat anti-mouse secondary antibody at a 1:5,000 (KPL). Signals were detected with an ECL PLus chemiluminescence reagent (ThermoFisher Scientific). The membrane was stripped between primary antibodies using Restore™ plus western blot stripping buffer (Thermo Scientific).

2.4 GPC3-hFc cell viability and ELISA assays

GPC3-hFc expressing cells were transfected as above. Seven days after transfection, the supernatant was collected and centrifuged for ELISA assay to measure the GPC3-hFc concentration. Cell counts and viability were determined using a CEDEX cell quantification system (Roche, Mannheim, Germany). The remaining cells were concentrated for RNA extraction.

A 96-well MaxiSorp high binding plate (Nunc) was coated with 5 μL/mL AffiniPure F(ab')2 Fragment Goat-anti-human IgG (Jackson ImmunoResearch Laboratories) in PBS 50 μL per well. After overnight incubation at 4°C, the plate was washed with PBS containing 0.05% Tween 20 (PBST) and blocked with 3% milk in PBS for 30 min at 37°C. Prediluted expression media, starting at 40-fold and using 1:2 serial dilutions, was added at 50 μL per well and incubated at 37°C for 30 min. After washing twice with PBST, 50 μL per well of 1:4000 dilution of Peroxidase conjugated AffiniPure Goat-anti-human IgG (Jackson ImmunoResearch Laboratories) in blocking buffer were added and the plate was incubated at 37°C for 30 min. The plate was washed 4 times with PBST, and TMB Microwell Peroxidase Substrate System (KPL) was used to detect quenching with 1 M phosphoric acid, after incubation for 5 min at room temperature. Absorbance was read with a SpectraMax i3 plate reader (Molecular Devices) at 450 nm. The amount of GPC3-hFc was determined by GPC3-hFc per cell production was quantified by comparing to a standard. The GPC3-hFc per cell production was determined by dividing GPC3-hFc by the viable cell number. Percentages were calculated dividing the result by that of the negative control and multiplying by 100.

2.6 RNA extraction

Total RNA, including microRNA, was extracted from the cell pellets of transfected cells with the miRNEasy kit with DNase Digestion (Qiagen), following the manufacturer's protocol, with an extra RPE buffer (Qiagen) wash. The extraction process involved lysing the cells and purifying with a spin column. RNA concentration and quality were determined with the NanoDrop 2000 Spectrophotometer (ThermoFisher Scientific) and Agilent 2100 Bioanalyzer (Agilent).

2.7 Microarray

Triplicates of luciferase-expressing cells transfected with either miR-22-3p or negative control were used to extract RNA for microarray analysis. The GeneChip Human Gene 2.0 ST array and the GeneChip WT Plus reagent kit (Affymetrix) were used to measure protein coding and long intergenic non-coding RNA transcripts. The Biopolymer/Genomics Core Facility at the University of Maryland performed the reverse transcription, hybridization and data collection for the microarray. For gene identification, the raw cell files from the microarrays were analyzed by using both Gene ANOVA and alt-splice gene ANOVA workflow of the commercial software Partek Genomic Suite (http://www.partek.com/pgs). Genes with a p-value less than 0.05 and absolute value fold change of at least 1.5 (Supplemental Table 1) were used.

2.8 nCounter XT CodeSet Gene Expression Assay

The RNA extracted from the miRNA-transfected luciferase-expressing cells was used to confirm gene expression with the nCounter analysis. A custom CodeSet (NanoString Technologies) was created for the 27 genes identified by the microarray. The RNA was hybridized with the CodeSet and ProbeSet following the manufacturer's protocol using 100 ng RNA. The data collection was performed by the CCR Genomics Core at the National Institutes of Health, with the automated processing nCounter instrument (NanoString Technologies). The raw data files were analyzed using nSolver. Genes were considered if they had a p-value less than 0.05.

2.9 qRT PCR

For microRNA expression analysis, miScript PCR starter kit (Qiagen) was used with the miR-22-3p miScript Primer Assay (Qiagen) following the manufacturer's instructions. Briefly, 100 ng RNA was transcribed to cDNA by incubating with the Reverse Transcriptase mixed with high flex buffer. Then the qPCR was performed using the SYBR green mix, primer assay and universal primer with the prescribed conditions and measured on the 7500 Fast Real Time PCR System (Applied Biosystems). Relative gene expression was calculated using the $2^{-\Delta\Delta CT}$ method with human RNU6B as the reference gene.

For gene expression analysis, RNA extracted from transfected cells was transcribed to cDNA using reverse transcriptase (Life Technologies) following the manufacturer's protocol: 100 ng of RNA were mixed with reverse transcriptase master mix and incubated at 37°C for 60 min followed by a 95°C incubation for 5 minutes. Primers against the genes homeodomain interacting protein kinase 1 (HIPK1), frequently rearranged in advanced T-cell lymphomas 2 (FRAT2), or Photinus pyralis (LUC2) were added in triplicate to SYBR green PCR master mix with glyceraldehyde 3-phosphate dehydrogenase (GAPDH) for normalization along with the respective RNA. The quantitative PCR amplifications were measured on the 7500 Fast Real Time PCR System (Applied Biosystems) with initial 10 min at 95°C followed by 40 cycles of 15 seconds at 95°C and 10 seconds at 65°C. Relative gene expression was calculated using the 2−ΔΔCT method with GAPDH as the reference gene.

3 Results

Work Flow

The work flow of the gene identification process is shown in Figure 1: Following transfection of the HEK cells with hsa-miR-22-3p, microarray analysis was performed and 208 down-regulated genes were identified. These down-regulated genes were compared with the list of the predicted targets [18] and 27 genes were selected and confirmed. Then, from the previously conducted genome-wide siRNA screening [7], 1,856 genes that showed above 60% improvement of luciferase expression (when inhibited by siRNA) were compared with the down-regulated genes obtained from the microarrays. Six genes were found to be included in both groups and were selected for follow-up siRNA studies.

3.1 Effect of hsa-miR-22-3p on protein expression

The effect of hsa-miR-22-3p on the expression of luciferase from HEK 293 cells was verified by transfecting the cells with the miR-22-3p. The luciferase expression, cell viability and western blot results are shown in Figure 2. The overall luciferase expression in the transfected cells was 3.7 times higher than the expression in cells treated with negative control. The western blot also shows increased luciferase protein in cells treated with miR-22 compared to the negative control. Following the transfection, the amount of miR-22-3p in the cells was measured using qPCR and was found to be 1038 ± 235-fold higher than the amount in the controlled cells.

3.2 Identification potential targets of miR-22-3p that involved in improved protein expression

A HuGene 2.0 microarray analysis, comparing RNA from HEK 293 luciferase-expressing cells transfected with miR-22-3p with RNA from cells transfected with negative control, was performed. The data have been deposited in NCBI's Gene Expression Omnibus [19] and are accessible through GEO Series accession number GSE92599. A one-way analysis of variance (ANOVA) of the microarray data of the 34,460 unique genes, identified 405 significantly differentially expressed (DEG) gene with p-value of less than 0.05 and a fold change greater than 1.5 or less than −1.5 (Supplemental Table 1).

Since miRNA typically regulate mRNA by repression, the 405-gene list was narrowed down to 218 significantly down-regulated genes with fold change of less than −1.5. To identify targets of miR-22-3p that are potentially involved in the increased luciferase expression, the 218 significantly down-regulated genes, were compared with the 430 predicted targets for miR-22-3p included in the miRDB database predictions by the MirTarget bioinformatics tool [18]. This comparison identified 27 genes that were included in both the down-regulated microarray list and the miRDB database; the genes are summarized in Table 1. To confirm the down-regulation of the 27 identified genes from the microarray, an nCounter (Nanostring) analysis was performed. This analysis verified that 26 of the genes were down-regulated with p-value less than 0.05 in luciferase expressing cells treated with miR-22-3p, compared with cells treated with a negative control (Table 1).

For further identification of the directly-involved genes the 27 down-regulated genes, that were also predicted targets of miR-22-3p, were compared with a subset of genes from a previously performed high-throughput siRNA screen that measured the effects of 64,755 individual siRNAs (representing 21,585 genes) on luciferase expression [7]. Since the miR-22-3p increased the luciferase expression, the 27 previously identified genes were compared to the siRNAs that improved luciferase expression by at least 60% in relation to the negative control; which created a list of only 1,856 genes from the siRNA high-throughput screen. Six of the 27 previously identified genes HIPK1, FRAT2, elastin microfibril interface-located protein 1 (EMILIN3), lin-7 homolog C crumbs cell polarity complex component (LIN7C), muscle-restricted coiled-coil protein (MURC), and pre-mRNA processing factor 38A (PRPF38A) were also found on this short list of siRNAs. These genes were selected for follow up studies.

3.3 Validation of miR-22-3p identified targets

To validate the miR-22-3p selected targets, HEK cells expressing luciferase and HEK cells expressing GPC3-hFc were transfected with siRNA against each of the six identified genes (Supplemental Table 2); the effect of their inhibition on both expression and viability is seen in Figure 3. Decreasing HIPK1 expression increased luciferase activity 3.2-fold and GPC3 expression 2.3-fold, while decreasing FRAT2 expression increased luciferase activity 3-fold with no effect on GPC3 expression. Decreasing expression of LIN7C and MURC increased modestly both luciferase and GPC3 expression, while decreasing expression of PRPF38A although modestly increased luciferase and GPC3 expression, has negative effect on cell viability.

Co-repression effect of HIPK1, FRAT2 and LIN7C, the top three siRNAs that were identified improving luciferase and co-repression effect of HIPK1, MURC and LIN7C the three-top siRNA that were identified improving GPC3 expression is shown in Figure 4. Co-repression of the luciferase expressing cells using siRNA against both HIPK1 and FRAT2 had a synergistic effect, increasing luciferase expression 4.4-fold, which is a larger improvement compared with siRNA inhibiting HIPK1 only (Figure 4A and B). Co-repressing HIPK1 with MURC or LIN7C in GPC3 cells increased the cell viability 1.9-fold and 2-fold respectively and the cell specific productivity 1.8-fold and 1.6-fold respectively which does not display a synergistic improvement. (Figure 4C). However, co-repressing MURC and LIN7C together in cells expressing GPC3 led to productivity increase of 2.5-fold compared with negative control which is improved compared to inhibiting MURC individually.

Additional confirmation that the increased recombinant proteins expression was associated with the decreased HIPK1transcription was obtained by quantitative reverse transcription polymerase chain reaction (RT-qPCR) analysis (Table 2). Cells treated with siRNA against HIPK1 showed 64% reduced transcription, and at the same time the luciferase gene transcription increased by 200%. An additional validation was done by using an nCounter analysis that confirmed that, when treated with the respective siRNA, the expression of HIPK1 was decreased and luciferase was increased. (Supplemental Table 3).

3.4 Off-target effects analysis

Off-target effects are frequent sources for false positives in RNAi screening [20]. To minimize the possibility that observed luciferase activity was due to seed-based off-target effects, each siRNA’s effect was compared to all other siRNAs from the screen having the same seed sequence (bases 2–7 or 2–8 of the guide strand of the seed sequence) [21]. By plotting the luciferase activity for the siRNAs designed against a given gene (three different siRNAs for each gene in the primary screen) along with the results for other siRNAs with the same seed sequence, an assessment was made as to whether the observed results were attributable to knockdown of the gene itself, or were the result of seed-based off-target effects. From this analysis, Life Technology’s Silencer Select siRNAs ID s47549 for HIPK1 (Supplemental Table 2) was confirmed as the most representative for true results.

4 Discussion

This work shows the benefit of using microarray analysis together with high throughput siRNA screen to investigate the effect of miR-22-3p on recombinant protein production from HEK cells. By using this approach is was possible to identify a possible target out of the 430 predicted targets genes that, when downregulated, enhanced productivity in two protein expressing cell lines. The findings demonstrated that HIPK1 is a predicted target gene of miR-22-3p, (Supplemental Figure 1); which is associated with increased recombinant luciferase expression, an intracellular protein and GPC3, a secreted protein. When cells were transfected with miR-22-3p mimic, HIPK1 was down-regulated while luciferase expression increased, and was confirmed by transfecting the cells with siRNA against HIPK1. Suppressing HIPK1with siRNA was also effective at increasing both the overall and per-cell expression of the secreted glypican-3 hFc-fusion protein, GPC3-hFc.

Recent studies demonstrate that mir-22 has different roles in different conditions. In the case of human glioblastoma [23], miR-22 inhibited proliferation [24] while in traumatic brain injury it prevented apoptosis [25]. The mature miR-22-3p is normally studied as part of general profiling of microRNA related to diabetes, hypertension atopic dermatitis and colorectal cancer [26–29]. There are few studies where, transcription factor 7 (TCF7) and regulator of G-protein signaling 2 (RGS2), gene targets of miR-22-3p were specifically differentiated from its counterpart miR-22-5p [30, 31]. Other targets of miR-22 are merely predicted with bioinformatics and have little experimental evidence to date [32]. Therefore, it is possible that by using the predicted target database, genes that are affected by miR-22-3p and improve protein expression will be identified but are not direct targets. Since microRNAs are known to directly target more than one gene at a time and can also indirectly target multiple additional genes, one of the goals of this work was to identify genes affected by miR-22-3p that are specifically associated with the increased expression of recombinant protein.

In previous high-throughput screen conducted in our lab [17], miR-22-3p was identified as a promising candidate for improving recombinant protein production, such as membrane proteins, a secreted fusion protein and an internal luciferase reporter. Since microRNAs target multiple genes, their overexpression can have unintended off-target negative effects. Identifying differentially regulated gene targets of the microRNA may lead to a better understanding of how the microRNA affects recombinant protein expression, with the possibility of minimizing off-target effects [11]. In the current study microarray analysis was used to identify the differentially regulated genes when the cells were treated with miR-22-3p. This traditional approach is an efficient method for identifying differences in gene expression; it requires less intensive bioinformatics analysis than RNA sequencing [33] since the probes are associated with known genes. Individually observing the effect of the down-regulated genes by knocking them down with siRNA is another way to determine which genes are involved in increasing the recombinant protein production. By comparing the list obtained from the microarray analysis to the results of the high-throughput siRNA screen and to the predicted list of targets, together with performing common seed analysis and verifying with siRNA and nCounter gene expression analysis, the HIPK1 gene was

selected. Using siRNA, the luciferase expression increased 3.3-fold when HIPK1 was inhibited and 4.4-fold when co-inhibited with FRAT2.

HIPK1 encodes the homeodomain interacting protein kinase 1, highly conserved member of the Ser/Thr family of protein kinases [34]. The main function of HIPK1 is to phosphorylate the homeodomain transcription factors' hydroxyl groups. This can have co-repressive effects on genes involved in transcription of RNA polymerase II which is responsible for transcribing mRNA and the small RNA precursors [34–37]. In addition, HIPK1 modulates different stress pathways implicating them in several types of cancer [38]. HIPK1 has been linked to apoptosis pathways through p53 [39, 40] and apoptosis signal-research regulating kinase1 (ASK1) [41], to growth pathways through mediation of death-domain associated protein 6 (Daxx) [42] and also has been found to be involved with the WNT/β-catenin signaling pathway regulating transcription, cell fate and cell proliferation [43].

Four of the six genes tested with siRNA improved expression of both luciferase and the secreted GPC3. In addition to HIPK1, these were LIN7C, MURC and PRPF38A. LIN7C is involved in activating calcium and potassium channels and combines with lin2 and the rest of lin7 to form the lin27 signaling complex [44]. MURC is a muscle-restricted coiled-coil protein that modulates the Rho/ROCK pathway and makes up caveolea in skeletal and cardiac muscles [45]. PRPF38A, pre-mRNA processing factor 38A, part of the human spliceosome, has a vital role in the cell and its repression with siRNA drastically decreases the cell viability [46]. While HIPK1, LIN7C, MURC and PRPF38A appear to improve both luciferase and GPC3expression, FRAT2 and EMILIN3 improved only luciferase expression. FRAT2 is Glycogen Synthase Kinase 3 (GSK3) binding protein that has a role in activating the WNT/β-catenin signaling pathway [47]. EMILIN3, elastin microfibril interfacer 3 is a member of the EMU gene family that interacts with extracellular matrix molecules and functions as an extracellular regulator of the of transforming growth factor beta (TGF-β) ligands activity [48]. Repressing FRAT2 and EMILIN3 likely acts in a more specific manner to increase luciferase expression thereby making them less useful for general protein expression.

Since microRNA functions by repressing multiple genes simultaneously, co-inhibition of the top three genes for both luciferase and GPC3-hFC expressing cells was carried out to determine if suppressing two genes would have a synergistic effect on protein expression. This does appear to be the case since simultaneously inhibiting HIPK1 and FRAT2 increased luciferase expression. However, the combination of HIPK1 and LIN7C did not have an improved productivity over HIPK1 individually. The combination of MURC and LIN7C, two genes that only had minor improvements in productivity of GPC3 individually had a large improvement when combined. Certainly, systematic high-throughput siRNA screen would be a more efficient way of determining which combination of genes complement each other for improving protein expression than drawing conclusions based on combinations of individual target genes.

4.1 Conclusion

The data presented here confirmed that HIPK1 is involved in the increased expression of recombinant luciferase and the secreted recombinant GPC3-hFc from HEK 293 cells

following transfection with miR-22-3p. As a result of exposing the cells to miR-22-3p, several genes are being down regulated, but the overall process that leads to the increased expression is not fully understood. Comparing microarray analysis with siRNA screening and performing common seed analysis was an efficient way to narrow the list of potential genes and to focus on a few that, when suppressed, significantly increased the specific productivity of luciferase and GPC3. A study of HIPK1 effects on recombinant protein production using a stable HIPK1 knockout cell line is in progress, expecting to provide an opportunity to explore more industrially relevant production format. It would be important to learn more about the mechanism behind the increased protein expression to find further improvements without affecting cell growth.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Abbreviations

CHO	Chinese hamster ovary
DEG	differentially expressed genes
EMILIN3	elastin microfibril interfacer 3
FRAT2	frequently Rearranged In Advanced T-Cell Lymphomas 2
GPC3	glypican-3 hFc-fusion protein
HEK	human embryonic kidney
HIPK1	homeodomain interacting protein kinase 1
HTP	high throughput
LIN7C	lin-7 homolog C, crumbs cell polarity complex component
LUC2	firefly luciferase also called Photinus pyralis
MURC	muscle-restricted coiled-coil protein
PRPF38A	pre-mRNA processing factor 38A

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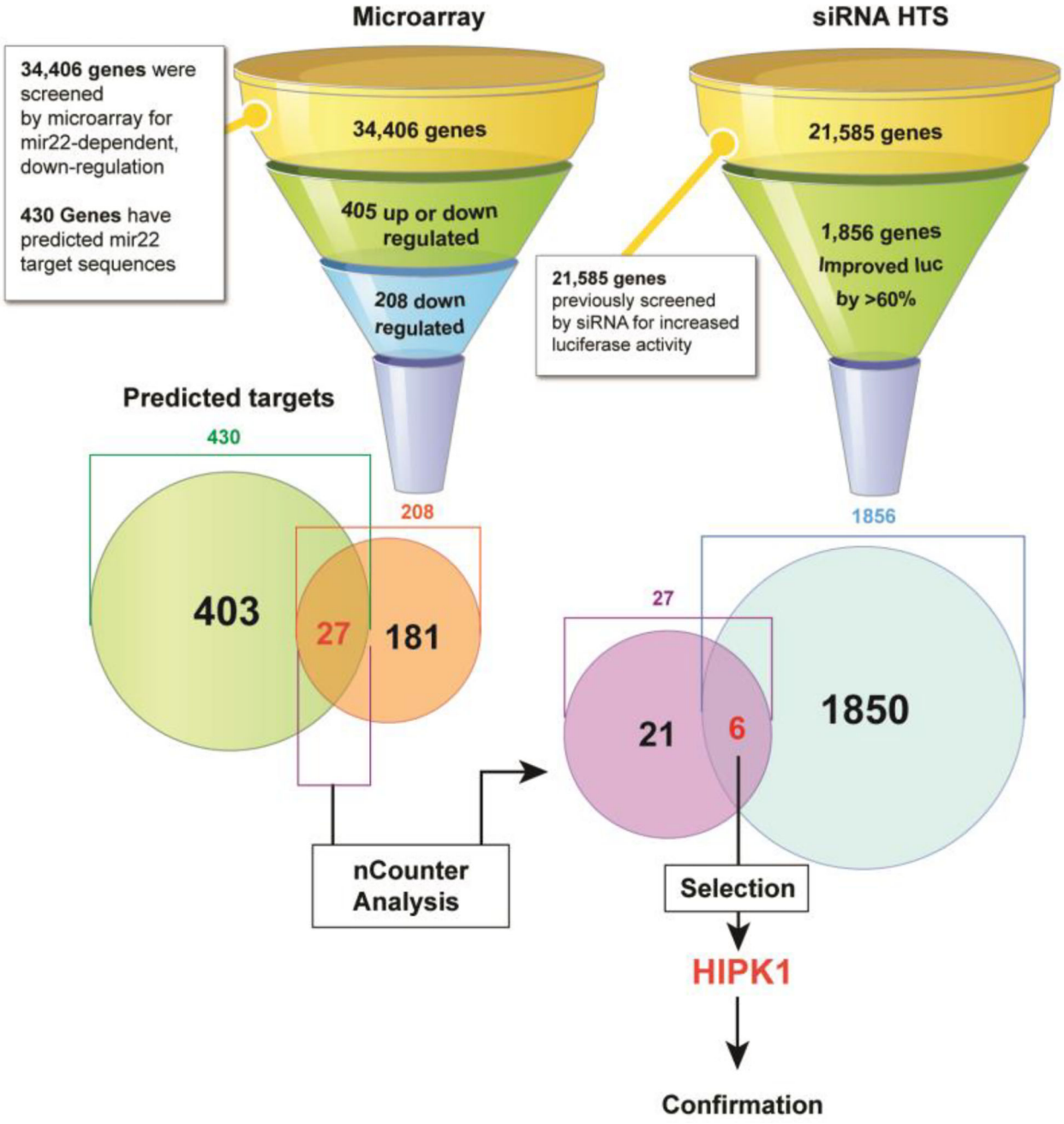

Figure 1. Work flow of identifying genes affected by miR-22-3p that improve recombinant protein expression utilizing microarray and siRNA screening

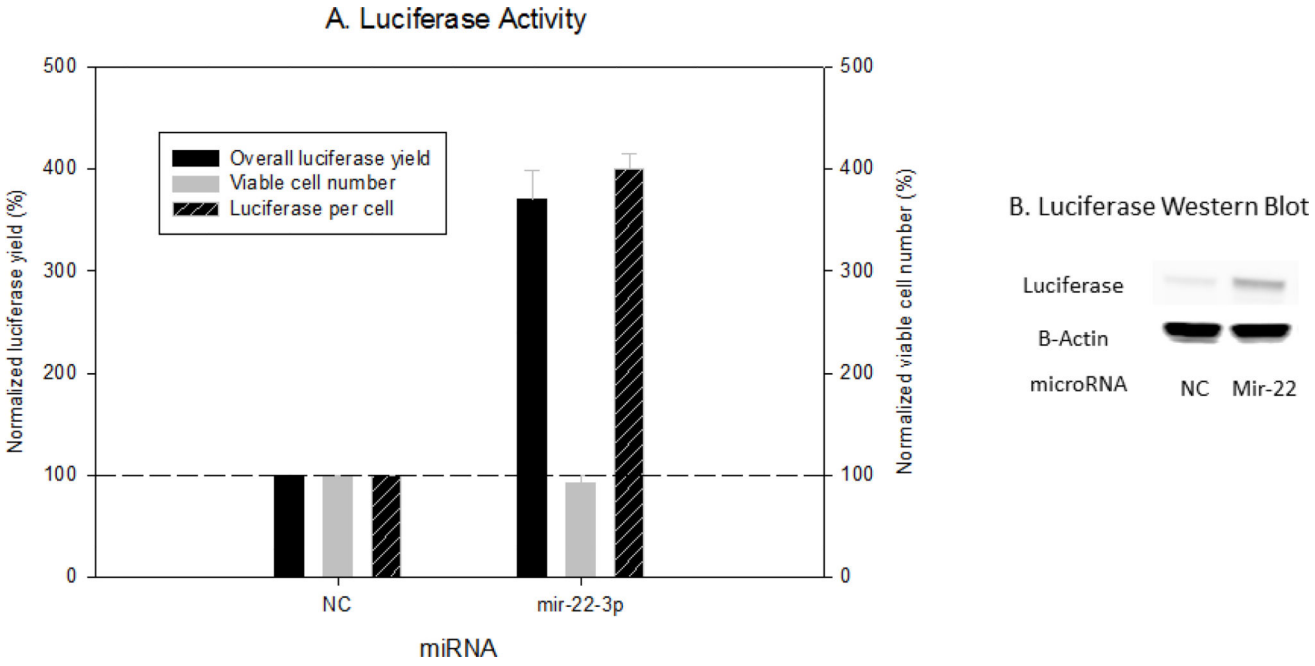

Figure 2. Effect of miRNA-22-3p on luciferase expression

(A) Overall luciferase yield, cell viability and luciferase per cell compared with negative control cells (NC). (B) Western blot of luciferase expressing HEK cells treated with miR-22-3p compared to NC. Experiments were carried out with biological triplicates Error bars represent Standard Error of the Mean (SEM).

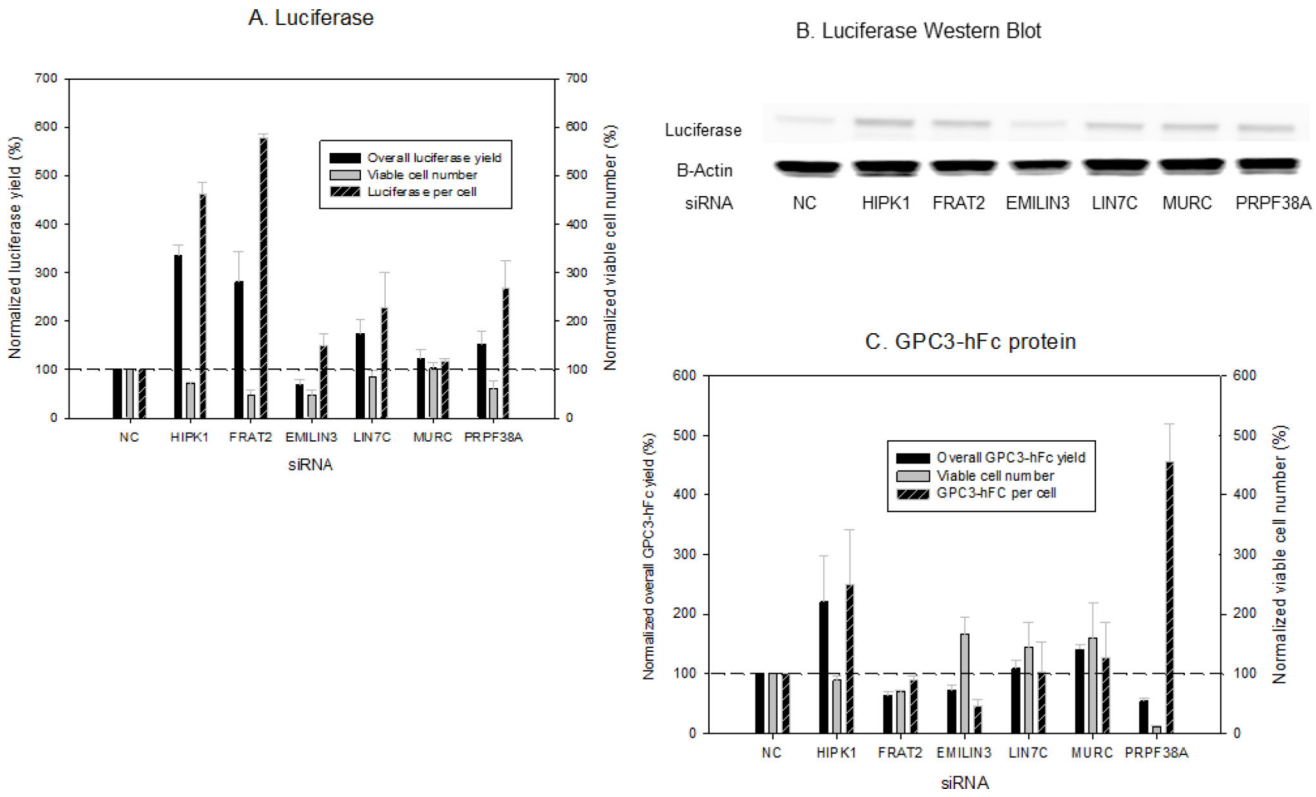

Figure 3. Effect of the elected siRNA on luciferase and GPC expression

(A) Relative luciferase yield, cell viability and luciferase per cell of luciferase expressing HEK cells treated with the selected siRNAs compared with NC cells. (B) Western blot of luciferase expressing HEK cells treated with the selected siRNAs. (C) Relative GPC3-hFc yield, cell viability and GPC3-hFc per cell of GPC3-hFc expressing HEK cells treated with the selected. Experiments were done with biological triplicates and error bars represent the SEM.

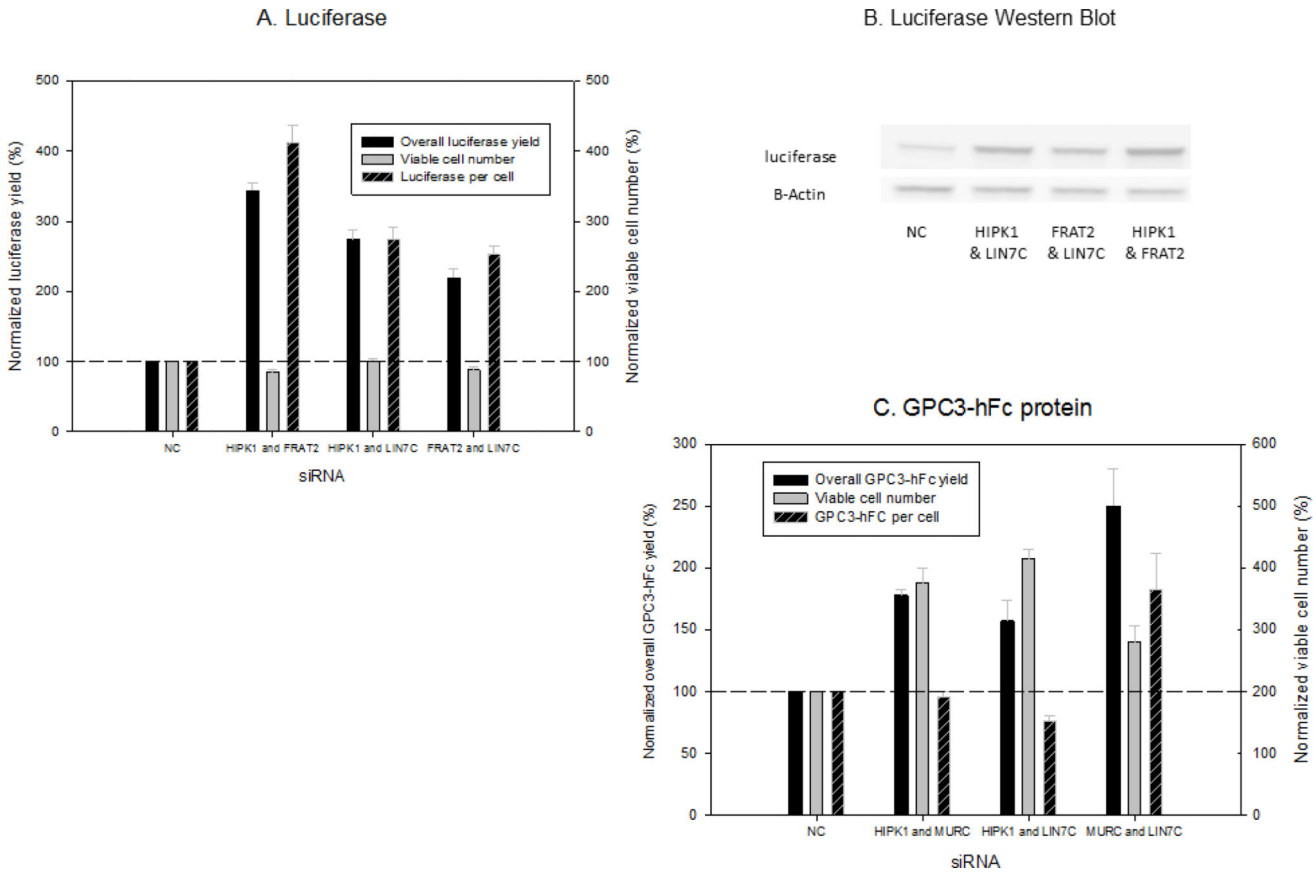

Figure 4. Effect of co-transfection of the selected siRNA on luciferase and GPC expression

(A) Relative luciferase yield, cell viability) and luciferase per cell of luciferase expressing HEK cells treated with 3 different siRNAs combinations (HIPK1 and FRAT2, HIPK1 and LIN7C, FRAT2 and LIN7C) compared with NC cells. (B) Western blot of luciferase expressing HEK cells treated with the same siRNA combination as A. (C) Relative GPC3-hFc yield, cell viability and GPC3-hFc per cell of GPC3-hFc expressing HEK cells treated with 3 different siRNAs combinations (HIPK1 and LIN7C, HIPK1 and MURC, LIN7C and MURC), compared with NC. Experiments were done with biological triplicates and error bars represent the SEM.

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Table 1

27 predicted miR-22-3p targets from the microarray analysis

Gene Symbol	Gene Name	Microarray P Value	Microarray Fold Change	Predicted Target Score	Nanostring P Value	Nanostring Fold Change	siRNA List
TP53INP1	tumor protein p53 inducible nuclear protein 1	0.0003	−2.01	93	0.0079	−2.64	Y
HOXA4	homeobox A4	0.0002	−1.99	82	0.0003	−2.31	Y
NUP210	nucleoporin 210kDa	0.0007	−1.72	59	0.0011	−2	Y
BRWD3	bromodomain and WD repeat domain containing 3	0.0002	−1.69	97	0.0006	−1.94	
SV2A	synaptic vesicle glycoprotein 2A	0.0025	−1.66	80	0.0118	−2.36	
H3F3B	H3 histone, family 3B (H3.3B)	0.0001	−1.65	93	0.0002	−2.04	
ELOVL6	ELOVL fatty acid elongase 6	0.0004	−1.64	97	0.0013	−2.26	
SMG7	SMG7 nonsense mediated mRNA decay factor	0.0003	−1.62	56	0.0022	−1.73	
RASSF3	Ras association (RalGDS/AF-6) domain family member 3	0.0006	−1.61	57	0.0003	−2.16	Y
ATP8A1	ATPase, aminophospholipid transporter (APLT), class I, type 8A, member 1	0.0002	−1.59	60	0.0002	−2.02	
MURC	muscle-related coiled-coil protein	0.0080	−1.58	77	0.2899	−1.27	Y
DPM2	dolichyl-phosphate mannosyltransferase polypeptide 2, regulatory subunit	0.0018	−1.58	73	0.0003	−2.32	
CBL	Cbl proto-oncogene, E3 ubiquitin protein ligase	0.0001	−1.58	61	0.0160	−1.65	
MOB1B	MOB kinase activator 1B	0.0026	−1.58	90	0.0014	−2.14	
UNK	unkempt family zinc finger	0.0012	−1.55	74	0.0210	−1.62	Y
PRPF38A	pre-mRNA processing factor 38A	0.0018	−1.54	87	0.0039	−1.77	Y
FRAT2	frequently rearranged in advanced T-cell lymphomas 2	0.0045	−1.54	68	0.0080	−2.45	Y
ACLY	ATP citrate lyase	0.0023	−1.53	60	0.0005	−1.67	
STK39	serine threonine kinase 39	0.0009	−1.53	79	0.0008	−1.69	
PHC1	polyhomeotic homolog 1 (Drosophila)	0.0016	−1.53	69	0.0005	−1.57	
EMILIN3	elastin microfibril interfacer 3	0.0222	−1.52	97	0.0193	−3.78	Y
NUS1	NUS1 dehydrodolichyl diphosphate synthase subunit	0.0007	−1.52	94	0.0022	−1.84	
SATB2	SATB homeobox 2	0.0001	−1.52	70	0.0002	−1.96	
LIN7C	lin-7 homolog C (C. elegans)	0.001	−1.52	92	0.0035	−1.39	Y
HIPK1	homeodomain interacting protein kinase 1	0.0003	−1.51	50	0.0031	−1.88	Y
ATP9A	ATPase, class II, type 9A	0.0022	−1.51	60	0.0007	−1.75	
GM2A	GM2 ganglioside activator	0.0062	−1.5	52	0.0218	−1.66	

Table 2

qRT-PCR of luciferase expressing cells treated with siRNA

HIPK1 expression		
Sample	**Fold difference**[a]	**range**[b]
Luciferase Control	1	(0.81–1.24)
siRNA against HIPK1	0.36	(0.30–0.43)
siRNA against HIPK1 and FRAT2	0.34	(0.32–0.36)
LUC2 expression		
Sample	***Fold difference**	****range**
Luciferase Control	1	(0.80–1.25)
siRNA against HIPK1	3	(2.71–3.33)
siRNA against HIPK1 and FRAT2	5.7	(5.40–6.01)

[a] Fold difference is siRNA relative to control calculated by $2^{-\Delta\Delta CT}$ with C_T of siRNA and C_T of GAPDH

[b] Range calculated from the standard deviation of $\Delta\Delta C_T$