JOHNS HOPKINS
UNIVERSITY
Department of Chemical & Biomolecular Engineering
3400 N. Charles Street; 221 MD Hall / Baltimore, MD 21218
410-516-5461 / Fax 410-516-5510/ beten@jhu.edu

Report

'Developing microRNAs as markers for stressed mammalian cells and further applications'

Lena Leberbauer

Absract

MicroRNAs (miRNAs) are short non coding hairpin shaped RNAs, that exhibit many functions within different cells. Their main function is to interfere with post-transcriptional protein expression steps, called RNA interference. Development of diseases have been linked to these small molecules, including cancer. As the requirement of recombinant therapeutic proteins rises globally with every year, every direction to increase production qualities and titers are pursued. Lately miRNAs are investigated intensely for this matter as their abundance within cells across all species are well known. Their potential to modulate whole biochemical pathways without burdening the metabolism, as other proteins like transcription factors or kinases do, could mean a highly advanced approach to engineer mammalian cells. Especially of the fact that eukaryotic cell systems are more complicated than prokaryotic ones and are therefore harder to modulate to obtain the desired effect, the nature of miRNAs, targeting multiple mRNAs due to partial base complementarities, can mean fast progresses. This works aim was to developed ways to find miRNAs that have promising effects on cell metabolism and to investigate their effects on the complicated cell network further, after their cellular miRNA pattern has successfully been modified. The objective to show that miRNAs can be markers for biochemical reactions as their modulation can change cellular properties and delay apoptosis, was pursued with different methods for reasons of simplifying future applications and create faster ways to success.

Introduction-

Mammalian cell cultures are commonly used to produce various biologicals used as biopharmaceuticals, which are already approved by the FDA. The production of recombinant proteins with mammalian cell culture has diverse advantages compared to the more economic use of bacterial cell cultures, as proper folding and authentic human- like post-translational modifications. Most important and frequently used cell types in biopharmaceutical production and research are Chinese Hamster Ovary (CHO) cells. Success for this cell line was granted when their properties of easy adaptability and plasticity. (Jadhav et al. 2013; Druz et al. 2013) Reaching high titers of recombinant proteins and enhance the productivity of engineered cells are the major goal of research nowadays. Different methods are used to accomplish this goal, but the main focus was on improving surrounding conditions during cell culture with improvement of media improvements and air composition. (Druz et al. 2013) Genetic engineering of cells to result in high productivity is a more difficult task due to the complexity of mammalian cell's biochemical and genetic systems.

MicroRNAs (miRNAs) belong to a group of short, endogenous RNAs with a length between 18 - 24 nucleotides with no known coding properties. Since the discovery in 1993, during genetic studies on nematodes, miRNAs have come a long way from unacknowledged short RNAs to powerful instruments, that are studied broadly for their implications on cell engineering and regulatory impacts on cellular pathways and gene expression (Jadhav et al. 2013; Wahid et al. 2010) MiRNAs are known to have a broad spectrum of functions across different species and cell types including cell growth and apoptosis, tumor development, cell differentiation and metabolism. Even more functions are expected to be influenced by these tiny single stranded RNAs. (Wahid et al. 2010) By now they are accepted as novel regulators that can control entire networks of genes and other cellular pathways and thus achieve a switch of cellular properties. (Barron et al. 2011; Hackl, Borth, and Grillari 2012) Understandably, researchers see high potentials in investigating these small molecules to their full extent .

The miRNA development starts in the nucleus. Following transcription, the primary RNAs (pri-miRNA) are enzymatically processed by a enzyme called Drosha to single stranded hairpin

shaped precursors (pre-miRNA). After being transported to the cytoplasm pre-miRNAs are enzymatically cleaved by Dicer, a protein that produces the final mature miRNA with a length of about 22 nucleotides. (Jadhav et al. 2012; Wahid et al. 2010) Subsequent loading into the so called RISC complex (RNA- induced silencing complex) activates the post-transcriptional inhibition function of miRNAs by being able to target the 3'- untranslated region of mRNAs due to partial complementary sequences and thus, inhibition of translation or by degradation of mRNA by a RISC subunit. The outcome is an interference of the cells gene expression known as RNAi (RNA interference).(Müller, Katinger, and Grillari 2008; Jadhav et al. 2012) Not only miRNAs belong to the mechanism that control the sophisticated system of gene silencing. Small interfering RNAs (siRNA) play another big role in gene silencing and occur naturally in cells. (RNA Interference (RNAi): By Nature Video 2011) Today, the magnitude of importance of these small molecules guiding genomic expressions and biochemical pathways can only be assumed. A recent paper proposed that that miRNAs not exclusively have inhibitory effects on protein translation but can also enhance their production by different effects. (Vasudevan 2012)

Preface-

Screening for potential miRNAs to enhance cellular protein production

Hundreds of miRNAs can be encoded by the genome of mammalian cells, including humans, giving scientists a big new field to be discovered and researched. All of this created reasons to look further into this issue and investigate miRNAs regarding different directions, with different cells. With this work the we aimed to show the versatility of miRNAs and proof that their engineering can create detectable alterations in cellular response and can therefore be defined as biomarkers. First approach was to establish a procedure to screen for miRNAS that actually have the capabilities to increase recombinant protein production. Due to the high number of different miRNAs, experimental scales have grow smaller to enable a cost efficient and high throughput system to get results fast. (Vidugiriene et al. 2008) In this case microarrays were used to predetermine potential effective miRNAs for enhancement of protein expression. Using GFP and Flow Cytometry as a follow up enables a subsequent analysis of miRNAs effects

on protein expression. In Flow Cytometry different detectors are responsible to detect various of target fluorochromes and measures the emitted light (Herzenberg et al. 2006). Here, Green Fluorescent Protein (GFP). Using GFP as a reporter protein has different advantages. On the one hand side GFP can rather simply be utilized to determine optimal transfection conditions due to the fast and easy assay and detection via Flow Cytometry, on the other hand, detected GFP intensity directly relates to the number of GFP mRNA copy number in cells, which, in turn, gives clues about expression levels of other proteins in cells, e.g. by developing fusion proteins in plasmids. This gives reporter proteins like GFP the power to visualize differences of gene expression patterns and properties in-between cell populations. (Vidugiriene et al. 2008; Soboleski, Oaks, and Halford 2005) Thus, the decision how to develop and proof effects of multiple miRNAs on protein expression levels *in vivo,* was easily made towards the powerful tool GFP.

Materials and Methods-

Cell culture of adherent 293T HEK cell line and determination of microRNA'S used

The cells used for this experiment, HEK 293T-GFP cells, which were transfected with pcDNA3.1/Zeo(+)GFplasmid carrying a GFP and a Zeocin resistance sequence, were kindly provided by Su Xiao, from the National Institution of Health (NIH). Cells were cultured in T-flasks in incubator maintained at 37°C and 5% CO2. DMEM media supplemented with 4.5mmol L-glutamine, 10% FBS and 200µg/ml Zeocin was used for cell culture. Cells were subcultured for at least 2 weeks after thawing before being used for miRNA transfection experiment. 10 microRNAs, tested with HEK 293T-GFP cells, were provided by NIH. Pre-selection of the 10 miRNAs was performed by Su Xiao at NIH. Briefly, a library of 875 known human miRNAs were screened for enhancing neurotensin receptor expression by transfecting them into cells, to filter for miRNAs that can most significantly enhance protein production.

Transfection and GFP expression analysis

Transient transfection was performed in 12-well plate format by using transfection reagent Lipofectamine® RNAiMAX (Life Technologies, Cat.No.: 13778-085) and human miRNA mimics (Qiagen). The optimized transfection condition has been determined by Su Xiao by varying miRNA concentration, transfection reagent concentration and the seeding cell density Briefly, miRNAs were added to each well of a 12 well plate. Lipofectamin®RNAiMAX was diluted in of Fetal Bovine Serum (FBS) free DMEM media and let sit for a few minutes; this mixture was incubated with the individual miRNAs for 20 to 40 minutes after adding it to each well. Cells were detached, washed and diluted to the ideal cell density; this suspension of cells was added to each well, to allow transfection of cells with each miRNA. The last two wells contained "killer" siRNA inhibiting cell viability and a negative control siRNA each. These two last wells were used as positive and negative control for the transfection efficiency, by checking the growth ratios.

Harvesting and Fluorescence Analysis

As soon as the transfected cells reached a confluency of around 70%, they were harvested and prepared for Flow Cytometry analysis to detect the green fluorescence intensity for each individual well by detaching cells followed by multiple washing steps with DPBS to remove all colored liquids and produce a single cell suspension, necessary for analysis.

The assay itself was conducted with a FACSCalibur (Becton Dickinson, US) using DPBS as sheath fluids and parameters were set to measure exactly 10 000 events for the preset gate for this cell type specific pattern scattering patterns. Excitation was accomplished by laser with a wave length of 488nm and a detector at 530/30nm was used to capture the light signal. HEK T-REx-293 cell line without any GFP expression, used as a negative control to determine and compensate auto-fluorescence of HEK 293 cell lines were analyzed in parallel with every assay to minimize daily variations by the machine itself and by cell properties and growth conditions.

At the last experimental run of this procedure the parental 293T HEK cell line without any miRNA transfected into it was analyzed additionally.

Raw data was eventually analyzed using Flowing Software, Turku Centre for Biotechnology, Finland. Gating was done manually and individually for each cell line and each run based on 'dot plot' displays.

Results

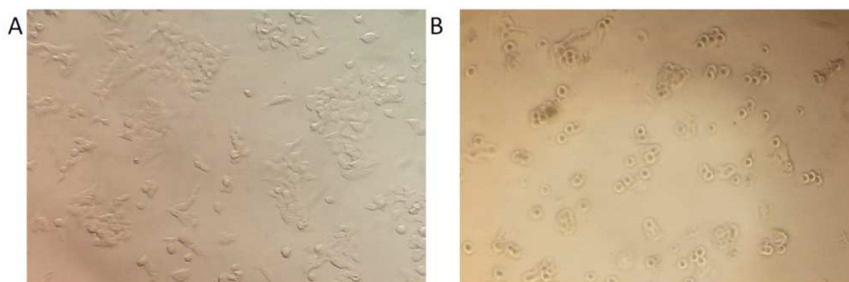

Figure 1: Comparison negative control siRNA and killer siRNA after 3 days. (A) cells transfected with negative control siRNA **(B)** cells transfected with the killer siRNA. Ratio of viability leads to the conclusion of effectiv transfection.

Transfection Step and Cell Culture

Cells were not thawed more than 2 weeks prior to transfection with miRNAs to keep the passage number low. If cells did not fit the viability requirements eventual findings were not included in final analysis. The transfection process with pcdna3.1 -GFP plasmid, created 12 different cell pools (Figure 2). Information about transfection efficiency was obtained with microsopical anlysis, see Figure 1. Picture A presents adherent healthy 293 HEK cells transiently transfected siRNA with no or little percentage of dead cells. B, on the other side, shows a very high ratio of cells that are already rounded up and floating around, not being attached to the surface. The later two runs of green fluorescent protein expression assessment, were

performed with a slightly adjusted protocol, reducing the incubation time of miRNAs with Lipofectamine RNAiMAX transfection reagent due to higher occurrence of apoptosis shortly after transfection. After slight modifications of the order of steps of transfection, performance of cell growth appeared normal again.

Flow Cytometry Analysis

An overview of the final results of all summarized runs is given Figure 2. Values of Geometric Means (GeoMean) and Medians were chosen to demonstrate the outcome as both of them are equally utilized to present data for cellular fluorescence quantification. Opinions which parameter has more statistical power are strongly divided, thus, both are presented in Figure 2. GeoMean is commonly preferred over arithmetic mean as the later one gives more statistical power to outliers and does not work well at describing Log amplified data. Therefore, GeoMean was chosen to demonstrate the GFP fluorescence intensity (Davies n.d.; Definition of Statistics n.d.; Reckermann 1998). Median is used, similar to GeoMean, as it does give outliers little or no influence on values and is considered at least as robust as GeoMean in describing a population of events (Best n.d.). The decision to present both parameters was made not to raise any questions but as the data shows, the relative difference between GeoMean and Median in comparing groups with each other are marginal and could be considered equal or at least very similar. In contrast to GeoMean, the Median seems to have the ability to create slightly more reproducible data, though. All results are scaled relatively to each other and do not display and absolute value. Green fluorescence intensity of the Blank, serving as a negative control for auto-fluorescence, is almost basal with both parameters, GeoMean and Median. Although the results within a 'group' (set of data for different miRNAs used) vary quite much from experiment to experiment, a certain trend can still be detected. Large amounts of cell debris were detectable but should usually be avoided to not interfere with data. Picture **D** should represent the variation in fluorescence intensity created by single miRNAs. Shift of GFP intensity can be seen on the x-axis which represents the GFP intensity. Considering the scale of the y-axis being cell count it is obvious that there is a shift of position of the peak (Mode) comparing both curves, demonstrating different effects of different miRNAs on cells.

One single attempt was made to actually use a different cell line for this line of experiment just to see if the procedure would work on other cell lines as well. Time was too little though to actually modify and optimize the transfection method for this cell line, being 293TRex HEK transfected with an inducible pcDNA4 plasmid carrying a fusion protein of eGFP sequence and serotonin construct. Some adoptions were made due to the cell line specific requirements as induction of protein expression with tetracycline, at a volumetric ratio of 1/1000, 48h after transfection and using a higher voltage of 485 at Flow Cytometry analysis. Results are presented in Figure 3. The pattern of GFP intensity and thus the related expression of the fusion protein is obviously varying from the first cell line, signaling that with different cell lines may come different results. The washing step caused a huge loss of cells with this cell line and has to be improved to receive results for all sets of transfected cells.

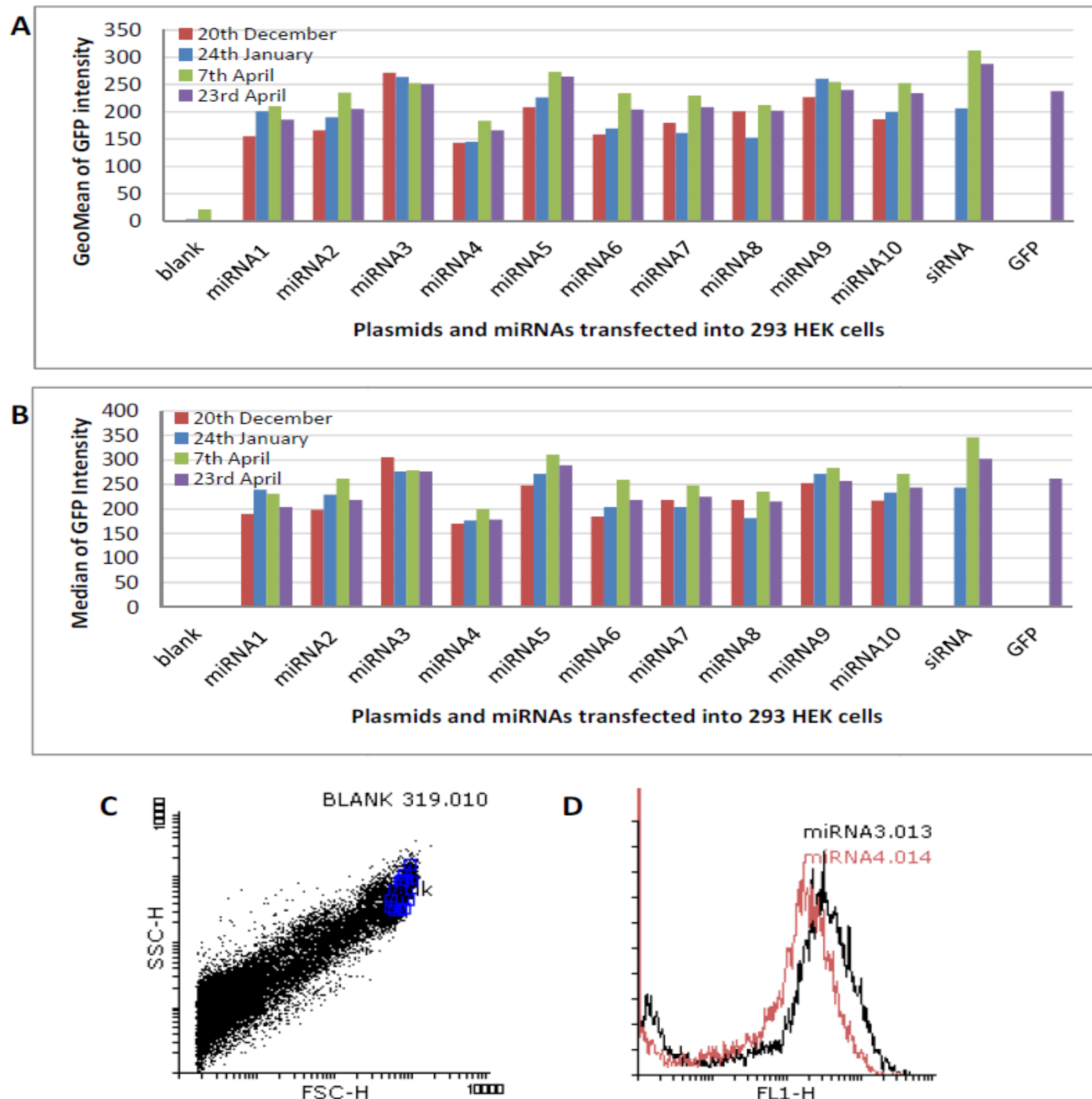

Figure 2: Overall View and comparison of Flow Cytometry (FC) Assay after Analysis. (A) Shows the GeoMean results collected from all evaluable runs of HEK 293 cell transfection with different miRNAs and subsequent analysis with FC. The ordinate (vertical axis) represent the relative GeoMean intensity of the GFP signal, colors represent different dates of the eventual FC analysis. The abscissa (vertical axis) is defined by cell modifications;blank: negative control (TREx-293 HEK cell line) which was whether transfected with a GFP expressing plasmid nor any kind of miRNA;GFP: 'parental' cell line used for this line of experiments containing a constitutively expressed GFP plasmid; miRNAs 1-10:each group was the result of transfecting the 'parental' cell line with different kinds of miRNA; siRNA: 'parental' cell line transfected with a scramble RNA with non-sense- coding. **(B)**Shows similar data with the only difference of the ordinate showing the Median fluorescence intensity **(C)** demonstrates one example (Blank) of the dot plots received at data analysis, with the gate around the 293HEK cell population, strikingly indicating a big count of dots excluded by the HEK 293 gate probably created by a large amount of cell debris. y-axis being Side Scatter and x-axis being Forward Scatter**(D)** Shows an overlay histogram of one miRNA transfected cell line with the highest fluorescence intensity (miRNA3) and one with lower fluorescence intensity (miRNA4). Scale of the y-axis is the cell count, x-axis represents fluorescence intensity.

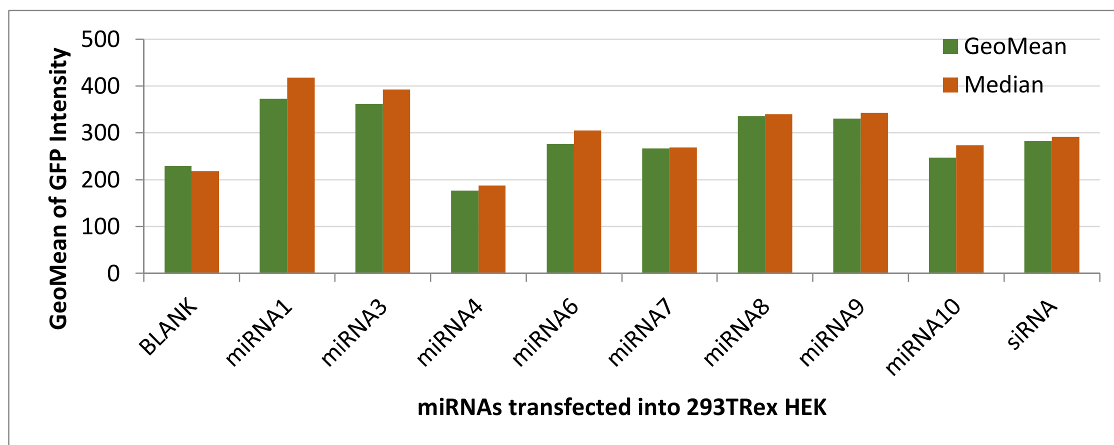

Figure 3: Results of GFP expression after miRNA transfection of 293-TRex HEK cells. Due to a single experiment GeoMean and Media could easily be summarized within one Figure. miRNA2 and miRNA5 cell pools are missing due to the loss of cell during the washing steps. miRNA cell pools 1 and 4 also have lower cell count in Flow Cytometry analysis compared to others.

Discussion-

Flow Cytometry, as an easy applicable method, was used to relatively quantitate GFP expression of cell populations stably transfected with the mentioned reporter gene. **(BD Biosciences Accuri C6 Personal Flow Cytometer - Applications - Gene Expression 2014)** By transfecting the varying miRNAs into the GFP expressing stable 293T HEK cell line, hope was to acquire different cell populations with different cellular properties, especially with GFP expression. Although a lot of research focuses on miRNAs nowadays, little is known about their individual effect on cellular pathway interference and subsequent change of cell systems. Proof that with this easy conductible experiment, aberrations in cellular components, system or metabolism can be achieved, would lay grounds for a more intense development towards this high throughput method to investigate a lot of miRNAs and their influence on cell types. Important here is that this could give an immense volume of additional information about complex cellular pathways, if promising findings would be followed up with assays of protein patterns or even analysis of metabolic fluxes.

A side effect of this line of experiments, to establish and improve the reproducibility of the GFP expression intensity patterns using different miRNAs for transfection and thus creating a

broadly useable and cell type comprehensive protocol, was not entirely achieved due to time and material issues. The plan to create a fully developed instruction to follow, which is also transferable to other cell lines and types and miRNAs, was also made more complicated by no reproducible cell culture conditions with certain reruns of the experiment. However, although the absolute values of GFP expression profiles within a cell population, transfected with a specific miRNA, e.g. all different values for miRNA1, are not comparable, it can be argued that there are certain trends within each group. One could claim that miRNAs three, five and nine seem to have and overall higher quality of green fluorescence protein expression. Especially compared to miRNAs four, six and eight, that seem to have the littlest intensity of GFP fluorescence. Due to the problems with cell culture conditions, time was too limited to apply further investigations of any independent miRNAs. One attempt to apply this scheme to a 293TRex HEK cell line were made. Again, problems with cell culture only allowed for one end result. Resulting in completely different patterns regarding miRNAs. Follow ups with this cell line and also others with more stable surrounding conditions could give the crucial results. Possibilities to find a miRNA that is worth pursuing to enhance protein production seems very promising. As a conclusion, further investigations on this matter are certainly necessary to definitely evaluate this approach of investigation. Most importantly standardization and constant cell culture conditions have to be given to go further otherwise fluctuations in results will most certainly occur again and time and cost investment will be in vain.

Preface-

Detecting Phenotypic and Genotypic changes caused by miRNA engineering of CHO cells

With publishing the paper 'Stable inhibition of mmu-miR-466h-5p improves apoptosis resistance and protein production in CHO cells' and thus, reporting a huge success with his research, Aliksandr Druz laid the basics for further investigation in the area of miRNA engineering and analysis, especially by pursuing his findings and explore the issue further. In his paper, Druz describes that the utilization of miRNAs in the approach of apoptosis engineering in mammalian cells might be more interesting and successful then the conventional approach in

manipulation a single gene and research its outcome. In previous studies Druz et al. investigated the miRNA expression patterns in Chinese Hamster Ovary (CHO) cells during apoptotic conditions, finding that a cluster of miRNAs (mouse miR-297-669) was up-regulated caused by bad nutritional conditions. Showing that, especially when inhibiting one member of this family of miRNAs (miRNA mmu- miR-466h-5p), which targets several genes with anti- apoptotic effects (dad1, bcl2l2, birc6, stat5a, smo), onset of apoptosis was delayed and cell viability increased. Briefly, after selecting the target miRNA Druz, A. created stable miRNA 466h-5p knockout cell clones, anti-miRNA-466h-5p, using short hairpin RNAs that target mature or pre-miRNAs of mmu-miRNA-466h-5p, due to base pair homology. Additionally, a negative control stable clone was created similar to the knockout cell line by using a short hairpin RNA with low homology to any relevant sequences. What he found was an equal growth rate of anti-MiRNAs466h-5p, negative control and non transfected cell line in the beginning of culture but an increased maximum viable cell density of the knockout cell line anti-miRNa-466h-5p, and similar maximum cell densities for negative control cell line and non transfected cell line, respectively. Also, he detected a light shift in time point of apoptosis onset with the anti-miRNA-466h-5p clones, again with similar results for negative control and non transfected, parental CHO suspension cell line. Investigation of possible enhancement of protein production using secreted alkaline phosphatase (SEAP) concluded in determination of higher total titers and cell specific productivity of SEAP especially in anti-miRNA-466h-5p engineered cells. It was mentioned that using miRNAs to create different intracellular properties has advantages as it might not have the same metabolic burden on cells due to less load on the translational machinery. This may give cells the opportunity to use more resources for recombinant protein production and lead to higher cell specific productivity, as seen in the results of Druz's study. (Druz et al. 2011; Druz, Betenbaugh, and Shiloach 2012)

This created curiosity what caused the higher protein yields and if and how the metabolism was altered and gained better properties for biopharmaceutical industrial use. Results lead to further investigations regarding glucose and other substrate consumption to explore the differences in-between these cell lines. However, findings needed to be repeated first to see if results can be recreated regarding growth and shift of apoptosis onset, in a lab

with different conditions. Furthermore, it was interesting to see if the anti-miRNA-466h-5p cell line could not only enhance the expression of the rather simple protein SEAP but also more complex protein that requires much more cellular resources.

As differences in metabolism of the three different cell lines are suspected a quick and easy experiment to confirm this theory was blissfully available as due to the presence of the Omnilog system. This system, which was kindly provided by Biolog (Hayward, CA), creates the possibility to investigate patterns of energy producing pathway in various cell lines, thus determining variations in-between cells. Basically, the system relies on dye reduction as a result of NADH production based on catabolism of biochemical substrates. Although, it is well known that media supplements like glucose, glutamine and pyruvate are big supporters of cell growth, a broader understanding of universal as well as unique potentials of energy production and substrate usage could not only be applicable to improve surrounding conditions of individual cell culture due to media modifications, but can contribute to a better understanding of differences in metabolic patterns after application of cell engineering and its resulting effects on cell properties. In our case, cell engineering resulted in a higher protein production but the reasons for this outcome are not known. If the modification due to the transfection resulted in a more effective metabolism and usage of energy of the anti-miRNA-466h-5p cell line, using the Phenotypic microarray system could deliver first indications of this hypothesis. Profiling cell lines can be conducted in a high throughput manner by seeding cells in microplates (PM M1). Wells are coated with different substrates, mostly carbohydrates and carboxylates, that could be metabolized if the biochemical pathways of the cell lines has the potential to. (Bochner et al. 2011; The Phenotype MicroArray Assay Technology For Optimizing Clone Selection, Cell Line Development, and Media Formulations in Bioprocessing - Biolog Inc. - Biolog Inc. n.d.)

Methods and Materials-

Cell culture and growth curve studies

Chinese Hamster Ovary (CHO) cells, that are adapted to suspension growth were kindly provided by Alex Druz at NIH, Bethesda, MD. Three different cell lines were received with

different passage numbers. Parental cell line (WT) that was previously purchased from Life Technologies, Gaithersburg, MD (Cat.no. 11619-012), exhibiting a passage number of 8, the negative control cell (NC), exhibiting a passage number of 9 and the anti-miRNA-466h-5p cell line (F9), exhibiting a passage number of 5. To culture the three suspension cell lines two different media were used, one being the chemically defined CD- CHO medium, Gibco®, Life Technologies (Ref. No.: 10743-011) and CD-OptiCHO^{TM} Medium (Ref. No.: ME120159L1) which did not contain L-Glutamine, Manganese, and D-GLucose. CD- CHO as a complete media could not be used for metabolic flux analysis due to the media already containing isotopically unlabeled Glucose therefore medium without Glucose had to be used, being, CD-OptiCHO^{TM} medium. Missing substrates were added by considering the optimal osmolarity of about 300mOsmo/L. The perfect amounts of 200g/L D-Glucose, 200mM L-Glutmaine, 300g/L NaCL and 10mM Manganese solutions to be supplemented were determined. Highest growth rate and highest cell viability was reached with a concentrations of 5g/L D- GLucose, 4mM L-Glutamine, 0,001mM Manganese and final addition of NaCl to reach an Osmolartiy of 300mOsmo/L. Therefore, this suspension was used for further analysis. Culture conditions for cell passaging and cell growth were kept stable 37°C, 5% CO_2, to simulate condition used by Druz, 2013. Cells grew on rotor shakers at 130rpm in 125mL glass shake flasks with the lid loosened to optimize air flow. Cells were seeded at a concentration of $1x10^5$cells/mL. Samples were taken daily and cells were counted manually by using a hemocytometer. Viability was determined by trypan blue exclusion method.

Glucose and Lactose concentration in Media

While taking samples for growth curves, concentrations of D- Glucose and Lactose were determined simultaneously with a YSI 2700 Select. Samples containing cells were centrifuged at 3000rpm for 10 minutes and supernatant was used for Glucose and Lactose concentration analysis.

Assessing varying substrate usage of different cell lines

For analyzing differences in cellular energy metabolism pathways in all three cell lines, an alternate assay was conducted. Biologs Phentype Microarrays provide a fast and simple method to simultaneously measure the ability of cells to use different substrates for energy metabolism.

Before preparing the cells for Omnilog's kinetic assay cells were cultured as previously mentioned. All three cell lines were prepared simultaneously in triplicates to perform the analysis. Cells were harvested and counted with hemocytometer. Cell viability was determined to guarantee ideal health for experimental conditions. Afterwards, cells were washed and resuspended to a density of 500.000cells/mL in Biolog's IF-M2 media, which was enriched with 0,3mM L-Glutamin but still lacked other amino acids and glucose. 50µL of this suspension was dispensed into each well of a 96well plate (PM M1) and subsequently incubated for 4 hours at 37°C and 5% CO_2, humidified incubator. After the incubation period, 10µl of Redox Dye Mix MB was added and immediately afterwards incubated in the Omnilog chamber to monitor Dye reduction, as a result of formation of purple formazan in the wells, was photographed for 4 hours every 5 minutes. (Bochner et al. 2011)

Using Microarray assay to determine apoptosis onset under different nutritional condition

Similar to the previous procedure with Omnilog's energy substrate usage analysis, all three cell lines were prepared by determining viability, washing with DPBS and seeding in Biolog specific media. However, other than before type of media, cell density, incubation time and dye was modified to result in different data provided by the system after analysis. IF M2 media was replaced by IF M1 media which contains the 20 amino acids that are lacking in IF M2 to avoid wrongful apoptosis onset due to the lack of necessary amino acids and guarantee apoptosis due to lack of primary energy giving substrates. IF M1 media was additionally enriched by adding 10% dialyzed FBS and 0,3mM L-Glutamine. Cells were resuspended to create a solution with a cell concentration of 40, 000cells/mL, meaning a total cell count of 2 000cells/well. A total of five PM M1 plates per cell line were seeded with these cell suspension and incubated at 37°C and 5% CO_2. Every 12 hours (adding up to a total of 48 hours), starting with zero hours (right after plating), 10µL/well of Redox Dye MB, containing 30mM of glucose was added to one plate

of each cell line and incubated in the Omnilog system at 37°C for four hours, detecting color change due to dye reduction. Using glucose in the dye at low cell seeding densities can spike the signal for better detection and will not or very little influence the results based on energy metabolism. This protocol was developed and conducted before for a Biolog intern study and created fairly good results for Hep G2/C3A cells.

Engineering non transfected, negative control and anti-miRNA cell line for protein expression

The gene encoding EPO was inserted into the plasmid pcDNA3.1 hygromycin, whereas the plasmid containing the HuBChe sequence was called pCHO 1.0. Backbones of both plasmids were received from Life Technologies. (A. Chung; Email communication, June 2014) EPO plasmid had to be amplified through E. Coli culture and subsequent plasmid purification following Qiagen® HiSpeed Plasmid Midid Kit procedure. Plasmids were transfected using FreeStyle™ MAX Reagent (Life Technologies, Cat. no. 16447-100) according to manufacturer's instructions in CD CHO medium. Speed of orbital shaker platform was reduced to 125rpm and CO_2 level was adjusted to 8% in the incubator to match the Life Technologies guidelines.

Cell lines transfected with EPO plasmid were cultured for 48 hours until complete harvest and suspension was centrifuged 2000g for 10min. Supernatant was obtained and stored at -20°C until further investigation with ELISA to assess EPO concentration in media. A total of two runs, from transfection with EPO plasmid to ELISA investigation, of the assay of EPO expression quality was performed. Before transfection and after harvesting cell counts and viability was determined.

Cell lines transfected with HuBChe plasmid were cultured for 5 days (120 hours). A total of four samples were taken on four consecutive days starting 48 hours after transfection. Samples were centrifuged at 1800g for 5min and supernatant was gathered for subsequent analysis with Ellman Esterase Assay with Ellman's Reagent (Thermo Scientific, No.22582). With each sample taken cell count and cell viability was determined.

Indirect ELISA and Ellman Esterase Assay to determine protein production

Abcam's® protocol for indirect Elisa was performed repeatedly to visualize and quantitate possible cell line specific EPO expression differences. Antigen containing supernatant of EPO transfected cell cultures was suspended in 96 well plates in different concentration and incubated for 12 hours overnight at 4°C. Negative controls were run in parallel. AntiEpo was used as a primary antibody in an 1:2500 dilution in 1% BSA in PBST solution and goat pAb as a secondary antibody in an 1:5000 dilution in 1% BSA in PBST solution. All washing steps were performed using PBST. 3,3’,5,5’-tetramethylbenzidine (TMB) was used as the visualizing reagent and its reaction was stopped by H_2SO_4. Colorimetric results were obtained by a plate reader (Promega) measuring optical density at 450nm. Best two dilutions were used to repeat analysis multiple times.

A single run of Ellman Assay was performed following the protocol of Thermo Fisher Scientific. The assay was conducted in 96 well plates in duplicates.

Results

Growth curve and Glucose and Lactate Yields

In parallel to the Omnilog's platform energy metabolism analysis, determination of glucose consumption yield and lactose production yield was measured with a YSI 2700 bioanalyzer. With every growth curve, glucose and lactose in the media was measured simultaneously. Considering viable cells, the Yield of cell count per glucose consumption and cell count per lactate production, respectively, was determined. Growth curves, glucose and lactose determination and yield calculations were performed with different medias resulting in varying outcomes regarding maximum cell density and lactose and glucose yields. A total of seven kinetic evaluations were performed with CD CHO medium, whereas two of them were not regarded based on their too high passage number and thus, possible kinetic changes due to mutation and another run was not evaluated due to sudden temperature switches and higher

rotation number of orbital shaker platform. A total of three kinetic investigations were conducted with CD Opti CHO medium, whereas two were supplemented to achieve a total glutamine concentration of 4 mM L-glutamine and one was supplemented to reach 8mM L-glutamine.

Statistical analysis of the data received from CD CHO media kinetics demonstrate that during exponential phase, the yield of amount biomass per amount glucose is higher in the anti-miRNA-466h-5p engineered cell lines compared to the non transfected one and especially compared to the negative control cell line, which resulted in the worst yield (data not shown). Maximum cell densities gave a similar profile, with anti-miRNA-466h-5p cells scoring the highest cell densities and negative control cell line the lowest maximum cell densities.

Definite differences between the two types of media are worthwhile mentioning. Cell densities were much higher in CDOptiCHO medium, universally with all cell lines, than in CD CHO media. Anti-miRNA-466h-5p cells reached a peak density of around 10 Mio cells/mL at compared to an average of 4,4 Mio cells/mL with CD CHO media. Other trends as yields and growth rates, regarding anti-miRNA-466h-5p in relation to other cell lines, stayed similar too. The data could not be validated enough due to the small sample size.

Metabolic fingerprinting Omnilog platform

The indications for possible different metabolic profiles were sought to be confirmed and Omilog's energy production assays were previously found to be effective to illustrate such distinctions. If changes in energy metabolism occurred due to transfection with anti-miRNA-466h-5p, quick results could be provided by the system. Profiles of all three cell lines were easily collected and are presented in Figure 4. After considering the kinetic background for calculations for the outcome, Dextrin, α-D- Glucose, D- Mannose, D- Galactose and D,L-Lactic acid still, had substantial growth curves to show for. Subsequent statistical analysis, including F-tests, ANOVA and students T-Test, using the initial rates of dye reduction and purple formazan production, lead to the following results: Significant differences between the non transfected cell line and the anti-miRNA cell line are exhibited with substrates α- D- Glucose and Dextrin,

whereas the differences in-between the anti-miRNA cells and the negative control only occur with D- Mannose. Other metabolites that demonstrated significant distinctions in-between all cell lines, e.g. D-Fructose-6-Phosphate or citric acid, were excluded of the data representation because metabolism in general was too low for comparison. (Bochner et al. 2011)

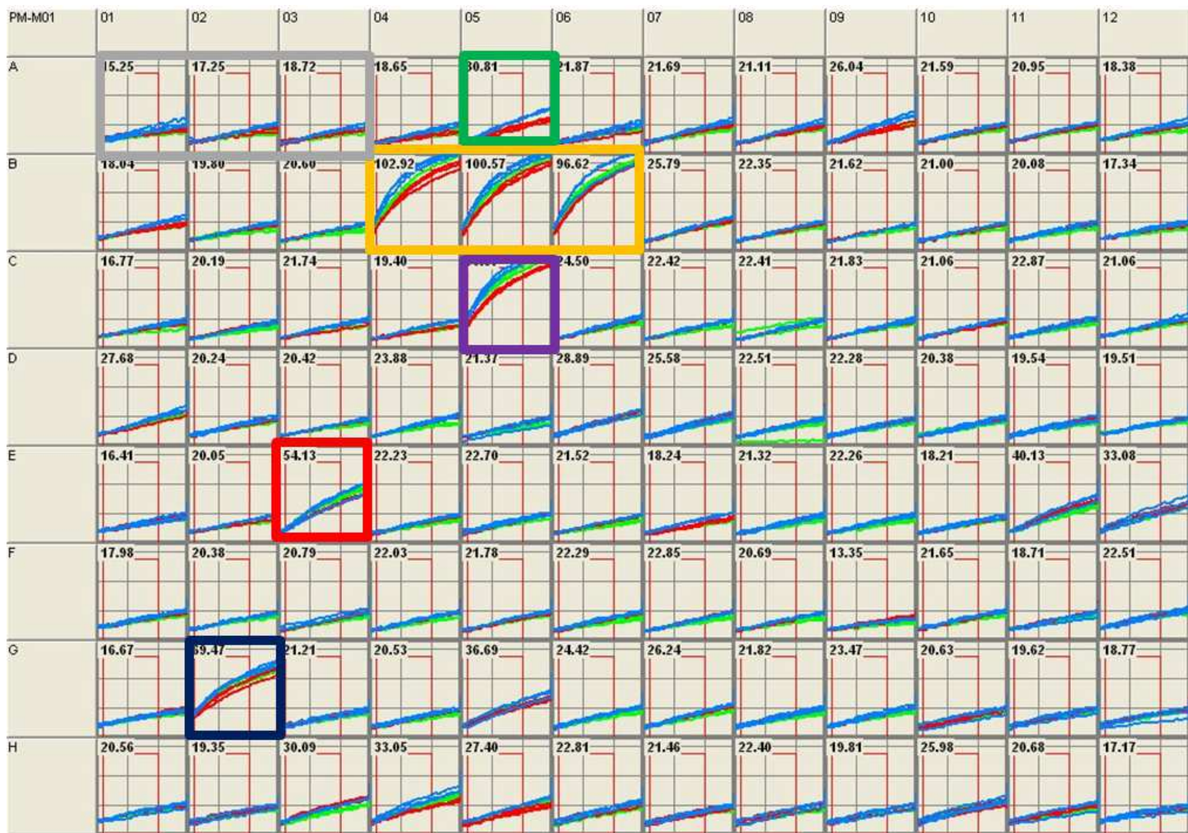

Figure 4: Kinetic graph of bioassay response of three cell lines in 96 well plates: Boxes (wells) are holding diverse substrates. Blue lines represent response of parental, non transfected cell line; red and green line show the negative control cell line and the anti-miRNA-466h-5p cell line, respectively. Numbers represent the initial rate of dye reduction. Substrates worth mentioning after subtracting kinetic background of negative control (grey frames) are Dextrin (green box), α-D-Glucose (yellow boxes), D-Mannose (purple box), D- Galactose (red box) and D,L-Lactic acid (dark blue box).

Proliferation, stasis, cell death via Omnilog metabolic profiling

Having the possibility to approach the apoptosis study in a different way, previous cell death investigations could be supported and confirmed. Additionally, cell death profiles on other main energy sources than glucose could be investigated, giving an even more accurate picture of

possible newly developed cell properties. Conducting this experiment was suggested by Barry Bochner, CEO of Biolog, Inc., to demonstrate the effect of different nutritional influences on apoptosis onset. Cells were incubated in Pehotypic MicroArray plates type one with no additional energy resource in their suspension solution, except for the substrates coated to the wells. The results of the first try of this experiment are presented in Figure 5. Different colors of lines count as different incubation times of the plates before monitoring the formazan production via Omnilog platform. Wells A1, A2 and A3 are negative control wells with no exogenous substrate added. Other wells contain various substrates.

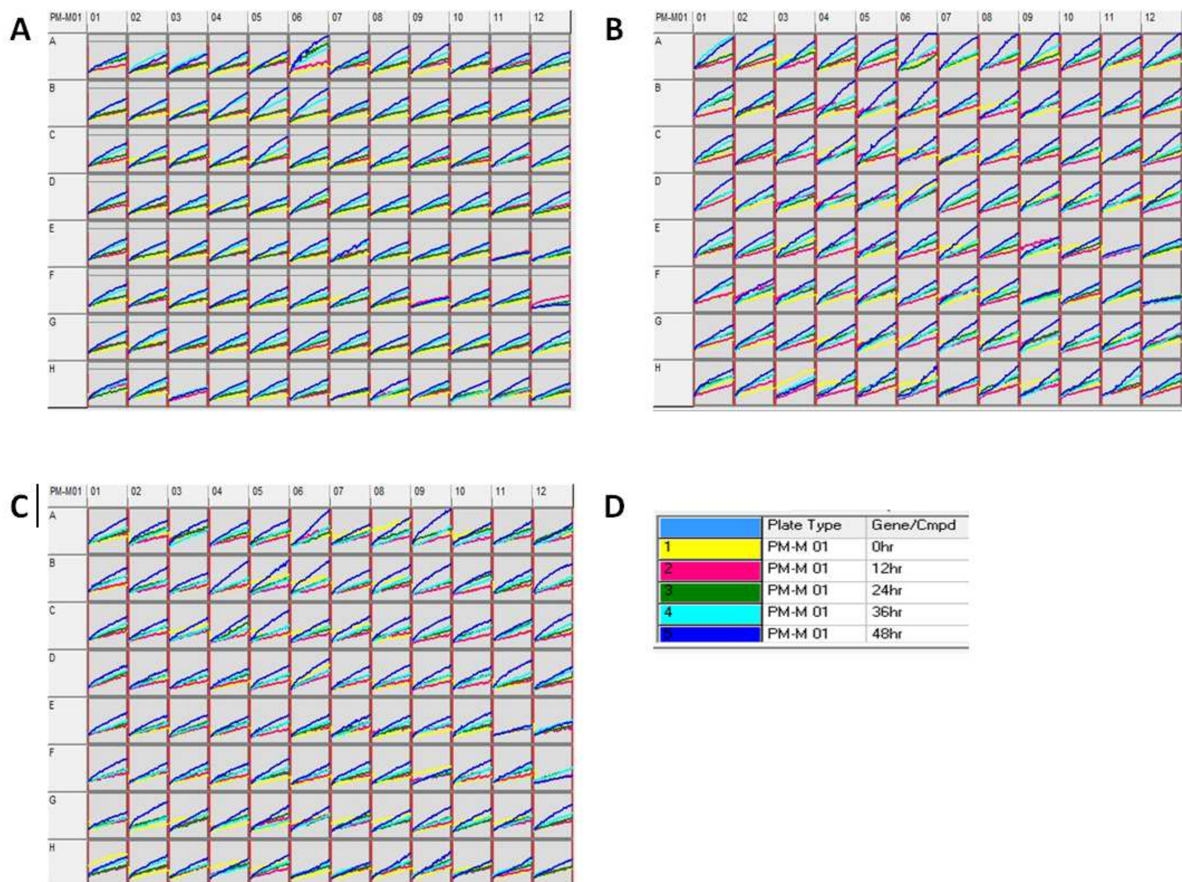

Figure 5: Growth and death assay. (A) Results for parental, non transfected CHO suspension cell line, **(B)** non transfected, negative control CHO suspension cell line **(C)** anti-miRNA- 466h-5p CHO suspension cell line. Color key of lines can be found in picture **(D).**

EPO and HuBChe production of CHO cell lines

Results for EPO and HuBChe expression differences can be explored in Figure 6 and

Figure 7, respectively. The expectations were that, based on results in . Druz's paper, anti-miRNA-466h- 5p should exhibit a higher expression of the protein EPO. Figure 6 shows the average results of all EPO determination assays, being supported by statistical evaluations that confirm the results presented as significant. Thus, the total EPO concentration of a cell population based the anti-mRNA- 466h-5p cell line is higher. This only shows a relative comparison though and does not count as absolute values.

Cells were counted before and at harvesting to establish the discrepancy in viability caused by transfection. Before transfection cells had a viability percentage higher than 95%. At harvesting aberrations could be detected in between each cell population. While the viability of anti-miRNA-466h-5p population was still high with ranging around 83% viable cells, followed by the non miRNA transfected cell line with around 72% and last the negative control cell line with about 65% viability.

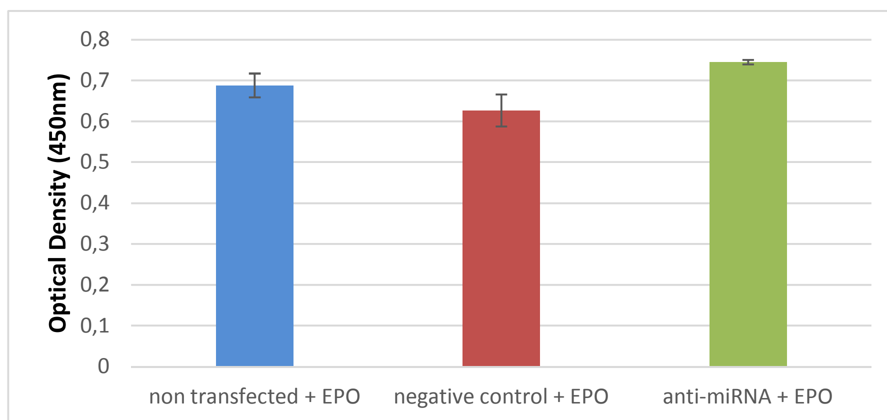

Figure 6: Results of indirect ELISA comparing EPO content of media. Height of the bars, representing different cell populations, show the level of EPO in the supernatant media.

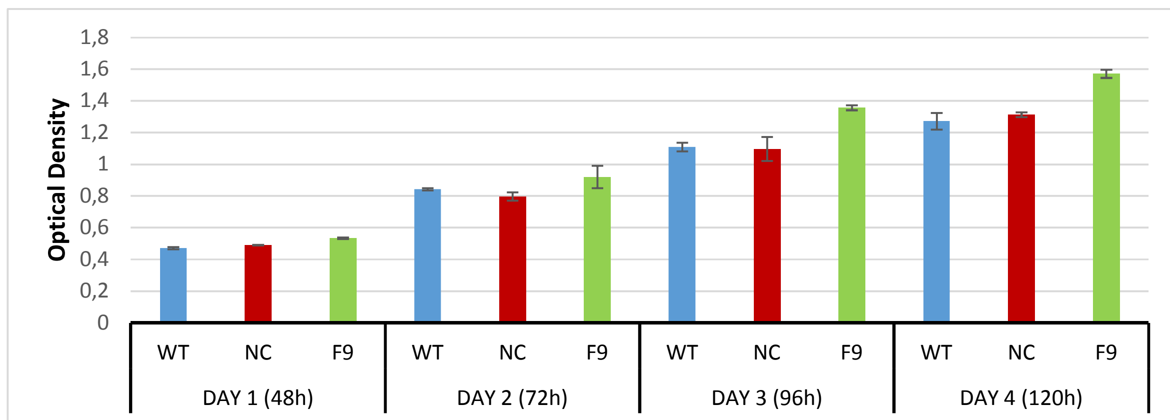

Figure 7: Differences of HuBChe expression levels within three cell lines. The figure shows the realtive amount of HuBChe in the media after transfection. x-axis exhibits the different cell populations as well as it demonstrated the harvest time after transfection. Each color represents a cell population. Blue color (WT) stands for the non transfected, parental cell line. Red color (NC) represents the transfected negative control cell line. Green (F9) is representative for the anti-miRNA-466h-5p cell population.

Discussion

Growth curve and Yields

From the beginning it was clear that reproducing A. Druz's results would proof to be difficult due to the different laboratory equipment and possibilities. Cells were counted manually, requiring more repetitions. Running the cultures in duplicates or even triplicates was not possible due to incubator capacity reasons. This way of procedure is much more prone to error, both by researcher handling and by surrounding conditions. Although, little can be said about

the absolute values of Yields and maximal cell densities, it could be determined that Druz's statement that anti-miRNA-466h-5p transfected cell line grows to higher densities and exhibits longer cell viability. (Druz et al. 2011), seems to be accurate Additionally, we found that growth speed tended to be faster, especially when shaker speeds were increased slightly; however, this would have to be confirmed by repetitions and stable surrounding conditions.

Yield determinations resulted in anti-miRNA- 466h-5p using less glucose per viable cell, which could be because of less effort of keeping cell densities stable due to longer cell life and less lactate production per cell. On the other hand, it was hypothesized that metabolism could have been changed and that glucose was used more effectively and therefore less energy was 'wasted' for higher gain of biomass and other metabolic pathways.

Surprisingly, cell densities in self supplemented media seemed to grow to higher absolute values and it raises curiosity if, when data can be validated, this could mean higher performances in recombinant protein production as well. This theory would be an interesting topic to investigate further. If combined with ideal investigations of media optimization using Biolog's Phenotypic MicroArray, where various substrates could show cell line specific usage profiles, higher protein titers could be yielded. Subsequently, a industrially scaled application could be investigated.

More analysis will be needed to give more statistical information. Due to the small sample size of comparable data, an actual validity and power of evidence is questionable. Trends can be mentioned nevertheless and these would support the statistical descriptions.

Metabolic profiling with Phenotypic MicraArrays

Specific miRNAs may modify and control the metabolic pattern of cells and therefore increase or diminish specific biochemical pathways within cell metabolism. Alterations regarding these miRNAs are linked to diverse genotypic and phenotypic changes and can eve cause serious diseases like cancer. (Druz et al. 2011; Druz, Betenbaugh, and Shiloach 2012) To see if the modification by stably transfecting a CHO suspension cell line caused aberrations from usual endogenous energy pathways, Phenotypic MicroArray was performed. Due to the procedure and materials used, by suspending the cells in 'energy-free' media, their only way of survival and

energy sources is the exogenous material provided in the individual wells. During metabolism energy is converted to mitochondrial reducing equivalents (NADH), if the cell can metabolize the very substrate that is provided, and colorimetric reactions can be seen and measured. (Biolog, Inc. n.d.)

Kinetic background can be created due to remnant energy from intracellular resources. After considering this kinetic background in statistical calculations, not many substrates give significant different values of energy metabolism. The suspected variations in Dextrin, α-D-Glucose, D- Mannose, D- Galactose and D,L-Lactic acid usage are not as big as assumed but can still be detected. Overall, one can hypothesize that negative control cell line has slower metabolic rate by interpreting results seen in Figure 4. Although, optimization runs were conducted to fine tune protocol steps to achieve optimal outcomes, more adjusting assays could lead to even better and more fitting protocols and better results. More trials are needed to gather further experimental data to be able to give proper scientific evidence.

Viability and Apoptosis assays using Biolog's Phenotypic MicroArrays

Previous presented Omnilog data indicates that changes in metabolic patterns can be explored easily without an abundance of complicated experimental steps, that could influence the overall investigation by being prone to mistakes. This creates new possibilities to achieve reproducible data. However, fingerprinting of metabolic pattern variations is not the only investigation that can be conducted with the kinetic Omnilog assay. B. Bochner and colleagues illustrated rather successfully that even proliferation, stasis and apoptosis can be detected with their phenotypic microarrays. By reducing the cell count seeded in each well, the well substrates will supply each individual cell longer with energy. Apoptosis can occur due to many reasons but nutrient depletion is one that most certainly will result in cell death. (Druz, Betenbaugh, and Shiloach 2012; Bochner et al. 2011) Provoking nutrient depletion by incubating cells up to a point when intracellular resources and energy of the wells should be consumed and metabolized will mimic energy depletion and cause cell death. Running this experiment in parallel with all three cell lines would have been able to give information to the individual cell

line apoptosis properties and resistance. In this case, however, the results are not what we hoped for. No real apoptosis was detectable after 48h of incubation. Although, in previous experiments with similar cell lines, the cell count and duration was ideal to gain proper results this time cells still seemed to be viable after the full incubation time and thus cannot provide information about differences in apoptosis onset in- between cell lines. (Bochner, B.; personal communication, April 2014) Thus, again, optimization steps to create favorable and reproducible results should be conducted before experiments are repeated and any distinct declarations can be made.

Protein production of EPO and HuBChe

Investigating the protein production with more complex recombinant proteins than secreted alkaline phoshphatase (SEAP) could give more information of the new possible metabolism capacities that may have been developed by transfecting cells with an inhibitor of a specific miRNA. If the metabolic burden was lowered as an implication of transfection, higher protein productions rates could also apply to more complex proteins like EPO or HubChe. What was discovered is an apparent overall enhanced production of target proteins in miRNA engineered cells but not with the negative control. Further investigation are needed to confirm these findings though. Additionally, cell specific productivity must be evaluated by taking the viability of cells after transfection into account. Pertinent arguments can only be made by confirming differences in metabolism with a detailed analysis. This can be done by performing a Metabolic Flux Anylsis (MFA).

Comment:

In addition it has to be mentioned that a Metabolic Flux Analysis was performed with the three cell lines but due to the time issues, the data could not be analyzed yet.The reason for this is that analyzing this raw data will cost much more time and raw data was received at the very end of the project. Final interpretations about intracellular changes in-between the three different cell lines can only be made after completing analysis on the MFA. Before that, theories about possible metabolic changes are still speculative.

References

Barron, N., N. Kumar, N. Sanchez, et al.
2011 Engineering CHO Cell Growth and Recombinant Protein Productivity by Overexpression of miR-7. Journal of Biotechnology 151(2): 204–211.

BD Biosciences Accuri C6 Personal Flow Cytometer - Applications - Gene Expression
2014 . https://www.bdbiosciences.com/instruments/accuri/applications/geneexpression.jsp, accessed June 21, 2014.

Best, Adam
N.d. Understanding MFI in the Context of FACS Data. Sanguine Bio Researcher Blog. http://technical.sanguinebio.com/understanding-mfi-in-the-context-of-facs-data/, accessed June 19, 2014.

Biolog, Inc.
N.d. Product Literature - Phenotype MicroArrays for Mammalian Cells - Biolog - Rapid ID of over 2500 Microbial Species - Biolog Inc. - Biolog Inc. http://www.biolog.com/products-static/phenotype_mammalian_cells_literature.php, accessed June 23, 2014.

Bochner, Barry R., Mark Siri, Richard H. Huang, et al.
2011 Assay of the Multiple Energy-Producing Pathways of Mammalian Cells. Daniel Tomé, ed. PLoS ONE 6(3): e18147.

Davies, Derek
N.d. Data Analysis. http://www.cyto.purdue.edu/cdroms/cyto3/8/data/icrf/stats.htm, accessed June 16, 2014.

Definition of Statistics
N.d. http://www.flowjo.com/v76/en/statdefinitions.html, accessed June 16, 2014.

Druz, A., M. Betenbaugh, and J. Shiloach
2012 Glucose Depletion Activates Mmu-miR-466h-5p Expression through Oxidative Stress and Inhibition of Histone Deacetylation. Nucleic Acids Research 40(15): 7291–7302.

Druz, Aliaksandr, Chia Chu, Brian Majors, et al.
2011 A Novel microRNA Mmu-miR-466h Affects Apoptosis Regulation in Mammalian Cells. Biotechnology and Bioengineering 108(7): 1651–1661.

Druz, Aliaksandr, Young–Jin Son, Michael Betenbaugh, and Joseph Shiloach
2013 Stable Inhibition of Mmu-miR-466h-5p Improves Apoptosis Resistance and Protein Production in CHO Cells. Metabolic Engineering 16: 87–94.

Hackl, Matthias, Nicole Borth, and Johannes Grillari
2012 miRNAs–pathway Engineering of CHO Cell Factories That Avoids Translational Burdening. Trends in Biotechnology 30(8): 405–406.

Herzenberg, Leonore A., James Tung, Wayne A. Moore, Leonard A. Herzenberg, and David R. Parks

2006 Interpreting Flow Cytometry Data: A Guide for the Perplexed. Nature Immunology 7(7): 681–685.

Jadhav, Vaibhav, Matthias Hackl, Aliaksandr Druz, et al.
2013 CHO microRNA Engineering Is Growing up: Recent Successes and Future Challenges. Biotechnology Advances 31(8): 1501–1513.

Jadhav, Vaibhav, Matthias Hackl, Juan A. Hernandez Bort, et al.
2012 A Screening Method to Assess Biological Effects of microRNA Overexpression in Chinese Hamster Ovary Cells. Biotechnology and Bioengineering 109(6): 1376–1385.

Müller, Dethardt, Hermann Katinger, and Johannes Grillari
2008 MicroRNAs as Targets for Engineering of CHO Cell Factories. Trends in Biotechnology 26(7): 359–365.

Reckermann, Marcus
1998 Mean, Geo-Mean, Median - Summary. https://lists.purdue.edu/pipermail/cytometry/1998-April/009771.html, accessed June 16, 2014.

RNA Interference (RNAi): By Nature Video
2011 . http://www.youtube.com/watch?v=cK-OGB1_ELE&feature=youtube_gdata_player, accessed June 22, 2014.

Soboleski, Mark R., Jason Oaks, and William P. Halford
2005 Green Fluorescent Protein Is a Quantitative Reporter of Gene Expression in Individual Eukaryotic Cells. The FASEB Journal : Official Publication of the Federation of American Societies for Experimental Biology 19(3): 440–442.

The Phenotype MicroArray Assay Technology For Optimizing Clone Selection, Cell Line Development, and Media Formulations in Bioprocessing - Biolog Inc. - Biolog Inc.

N.d. http://www.biolog.com/products-static/phenotype_mammalian_cells_bioprocess_improvement.php, accessed June 22, 2014.

Vasudevan, Shobha
2012 Posttranscriptional Upregulation by MicroRNAs. Wiley Interdisciplinary Reviews: RNA 3(3): 311–330.

Vidugiriene, Jolanta, Trista Schagat, Denise Garvin, Gediminas Vidugris, and others
2008 Getting the Most from Your Transfections: Increasing Throughput and Sensitivity. Signal 1(10): T14.

Wahid, Fazli, Adeeb Shehzad, Taous Khan, and You Young Kim
2010 MicroRNAs: Synthesis, Mechanism, Function, and Recent Clinical Trials. Biochimica et Biophysica Acta (BBA) - Molecular Cell Research 1803(11): 1231–1243.