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DESIGN OF A DUAL PLASMID SYSTEM FOR OVER-EXPRESSING MICRORNAS RELATED TO BREAST CANCER

A Major Qualifying Project Report

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ABSTRACT

MicroRNAs (miRNAs) are non-coding double stranded RNA molecules known to regulate gene expression. The recent discovery that the miR-30 family of miRNAs overexpress in mouse breast cancer tumor cells in the same regions commonly seen in human breast cancer tumor cells, has lead us to design a system for over-expressing these miRNAs to help evaluate their role in tumorigenesis. In this study, genes encoding miR-30c_1, c_2, a, and d, were successfully cloned. The gene for miR-30c_1 was further investigated in a tet inducible expression system. Fluorescence data indicated HEK-293 cells were successfully transfected with expression plasmid, and qRT-PCR on HEK-293 RNA indicated miR-30c_1 levels increased.

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BACKGROUND

MicroRNAs: Structure and Function

MicroRNAs (miRNAs) are recently discovered small non-coding RNAs that are 21-23 nucleotides long. The majority of miRNA genes are located in introns of protein coding genes (PCGs) or in intergenic non-protein-coding transcription units (Lee et al., 2004). They were first described as two small *lin-4* transcripts of approximately 22 and 61 nucleotides, that were identified in *C. elegans* and found to contain sequences complementary to a repeated sequence element in the 3′ untranslated region (UTR) of *lin-14* mRNA, suggesting that *lin-4* regulates *lin-14* translation via an antisense RNA-RNA interaction (Lee et al., 1993). These small RNAs were found to function in the down regulation of the lin-14 protein. At first they were called small RNAs, and only in 2001 were they named miRNA for their small nucleotide structures.

miRNAs are formed initially in the nucleus by RNA polymerase II transcribing the miRNA gene **(Figure-1)**, which creates primary miRNA (pri-miRNA), a 33 nucleotide stem structure and a terminal loop (Lee et al., 2004). This 70 base pair stem-loop structure is then cleaved by a Drosha-DGCR8 complex which creates the 70 nucleotide long pre-miRNAs. The DGCR8 group also known as Pasha can specifically identify and bind to pri-miRNAs (Rana, 2007).

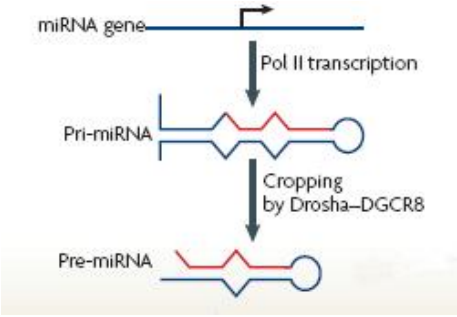

Figure-1: Transcription of miRNAs. miRNAs are initially formed in the nucleus by RNA polymerase II to create a primary miRNA (pri-miRNA) (diagram lower) (Lee et al., 2004).

Once the pre-miRNAs are created, they are transported out of the nucleus by a nuclear transport receptor complex, and are then received by Dicer in the cytoplasm **(Figure-2)**. Dicer cleaves the stem-loop into ~22 nucleotide long mature miRNAs. The miRNAs are then loaded on to the RNA-induced silencing complex, RISC, which alludes to one of the main functions of miRNA, gene-silencing (Chendrimada et al., 2005; Rana, 2007). The RISC complex is a multi-protein complex that uses siRNA as a template to degrade the sense strand, leaving an antisense miRNA. Once a miRNA binds its complementary mRNA, it cleaves the mRNA with RNase thus silencing its expression into protein (Zamore et al., 2000).

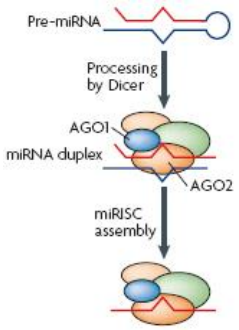

Figure-2: Formation of Mature miRNAs. Following transcription, pri-miRNAs are transported out of the nucleus and are received by Dicer that cleaves the stem-loop into ~22 nucleotide long mature miRNAs.

After being transferred onto the RISC complex, the miRISC identifies a matched target gene and the miRNA binds to the 3' UTR of the gene. The portion of the miRNA that binds to the target gene is known as the seed region. The seed region is 2-8 nucleotides long and dictates how families of miRNAs are formed. When a miRNA binds to its target gene in mammals, translational repression occurs in which translation is blocked and mRNA is conserved. In plants it is more commonly seen that miRNA cleaves mRNA and the mRNA becomes degraded (Bartel 2004).

Some of the other functions found for miRNAs are cell proliferation, development, metastasis and apoptosis. Due to these additional functions, recent research has focused on their role in oncogenesis and tumor suppression.

Role of MicroRNAs in Cancer

Some miRNA families have been proven to be associated with specific cellular functions, such as control of cell proliferation (*miR-125b* and *let-7*), hematopoietic B-cell lineage fate (*miR-181*), B-cell survival (*miR-15a* and *miR-16-1*), brain patterning (*miR-*

430), pancreatic cell insulin secretion (*miR-375*), and adipocyte development (*miR-143)* (Harfe, 2005). Those miRNAs that stimulate cell proliferation may also be associated with cancer.

Some miRNAs have been studied for their tumor suppressor and oncogenic qualities, such as miR-21, which is considered an oncogenic miRNA. It has been found to be overexpressed in breast cancer. Some miRNAs are also associated with more than one type of cancer, as in the case for miR-21 which was also discovered to be elevated in glioblastoma, VMP1 neuroblastoma primary tumors, and tumor cell lines (Calin and Croce 2006). Two families of miRNA that have been discovered to be tumor suppressors are the miR-16-1-15a cluster which is down-regulated or completely deleted in B-cell chronic lymphocytic leukemias (Negrini et al, 2007). Figure-3 shows a representation of miRNAs acting as tumor suppressors and oncogenes.

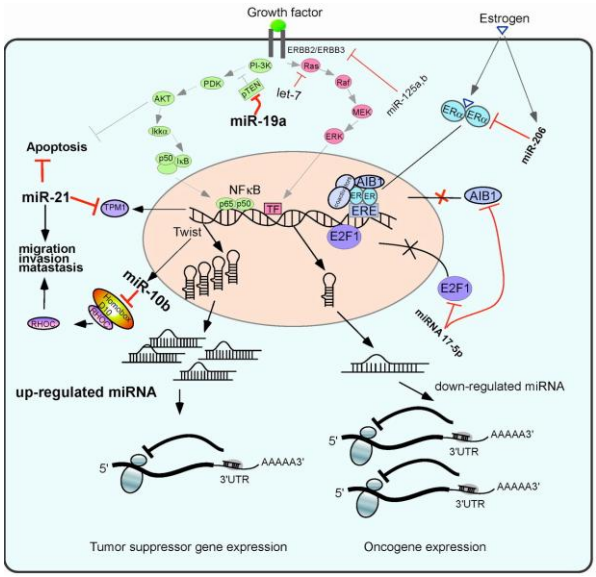

Figure-3: Diagram of Several miRNA Families Acting As Tumor Suppressors. (Wang and Wu 2007).

miRNAs and protein coding genes interact with each other to either form tumor suppressor proteins or oncogenic proteins. The abnormalities found in cancer to influence the activity of miRNAs are the same as those described to target PCGs. Inactivation of tumor suppressor PCGs and activation of oncogenic miRNAs cause reduced levels of proteins blocking proliferation and activating apoptosis. Activation of oncogenic PCGs and inactivation of suppressor miRNAs are followed by accumulation of proteins that stimulate proliferation and decrease apoptosis (Calin and Croce, 2006).

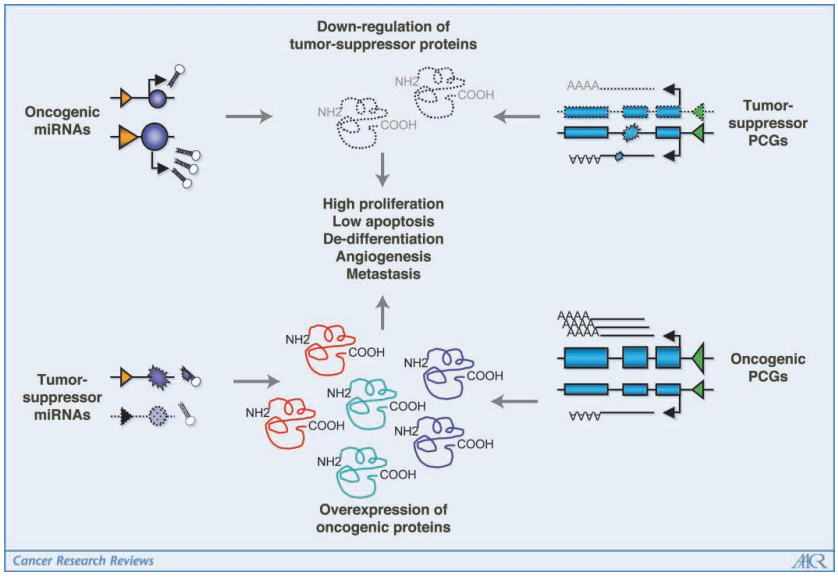

Figure-4: MicroRNAs Can Be Tumor Stimulators or Repressors. Shown is a comparison of the role of miRNAs as tumor stimulations (diagram top) or tumor suppressors (diagram lower) (Calin et al., 2006).

Role of MicroRNAs in Breast Cancer

The cancer of particular interest for this project is breast cancer, since it is one of the leading causes of death in women. Breast cancer occurs 100 times more in women than in men. Some of the causes of breast cancer are mutations in the BRAC1, BRAC2, or TP53 genes which are normally tumor suppressor genes, or the presences of the HER-

2/ErbB2 oncogene. ErbB2 is an estrogen receptor whose presence is an established breast cancer subtype. The degree of breast cancer is heavily dependent on amount of tumor suppressor genes the person has that are not mutated.

The findings that miRNAs have a role in human cancer is further supported by the fact that >50% of miRNA genes are located at fragile site regions, and regions of deletion or amplification that are genetically altered in human cancer (Iorio et al., 2005). It has also been recognized that miRNA expression might lead to the initiation of cancer becoming malignant, in that overexpressed oncogenic miRNAs are located in amplified regions found in tumors, and down-regulated suppressor miRNAs are located in regions deleted in cancers (Calin and Croce 2006).

In one study, miRNA microarrays were utilized to observe miRNA expression profiling in a large set of normal and tumor breast tissues demonstrating the existence of a breast cancer–specific miRNA signature. The levels of miRNA in normal tissue and breast carcinomas were analyzed to confer that multiple miRNA families are involved in breast cancer progression (**Figure-5**) (Iorio et al., 2005). The expression levels of let-7a, miR-10b, miR-125a, miR-125b and miR-145 are higher in normal epithelial cells relative to breast tumors; whereas, miR-21, miR-155, miR-206, miR-122a, and miR-210 are higher in breast tumors compared to normal tissues (Wang and Wu, 2007).

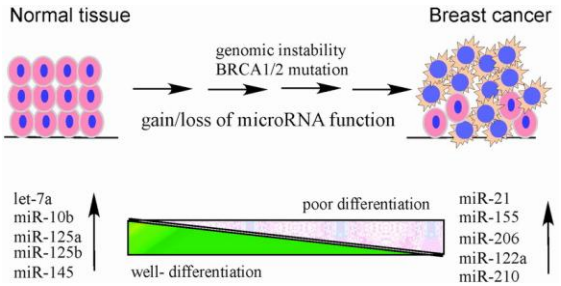

Figure-5: Key Candidate miRNAs in Breast Cancer (Iorio et al., 2005).

However, most miRNAs are expressed at lower levels in breast tumors than normal tissues. Moreover, miRNA levels are lower in poorly differentiated breast tumors compared to highly differentiated breast tumors. As mentioned before, the miRNA profile is more accurately correlated with cell differentiation. By comparing the differentiation levels of miRNA in tumor cells, it can be speculated that their levels are affected by methylation and demethylation of miRNA promoter regions during cancer development. However the miRNA may mutate in breast cancer, which may affect the detection of expression. Thus genomic instability, epigenetic change, and mutation of miRNA all cause miRNA dysregulation in breast cancer (Wang and Wu, 2007).

Another correlation has been found between subsets of miRNA expression and estrogen receptor expression. Especially in determining ErbB2-positive/ER positive and ErbB2-positive/ER-negative breast cancers. This can become a useful diagnostic tool for prognostic biomarkers for breast cancer (Wang and Wu, 2007).

One of the target pathways of miRNAs is the TGF-β signaling pathway. This inhibitory pathway is involved in inducing cell-cycle arrest in part through suppression of c-Myc-mediated gene expression. c-Myc is a well known breast cancer oncogene, and

the ectopic expression of c-Myc nullifies TGF-β-mediated growth inhibition. It is hypothesized that overexpression of miR-21 inhibits the TGF-β gene, diminishing the inhibitory effect of TGF-β signaling, enhancing cell growth, and promoting breast cancer development (Wang and Wu, 2007).

The miR-30 Family

The family of miRNAs chosen for this project is the miR-30 family. miR-30s are all down-regulated in both estrogen receptor-negative and progesterone receptor–negative tumors, suggesting that expression of these miRNAs is regulated by these hormones (Iorio 2005), and suggesting they bind to the mRNAs of cell proliferation proteins to decrease their expression in normal cells. Due to this family's links to ERbB2 receptors and the p53 tumor suppressor (Sachdevaa et al., 2009), further investigation of this family of miRNAs was initiated in the Simin lab breast cancer mouse models. This miR-30 family was also picked for the genes it targets which are involved in cell proliferation and metastasis and its accumulation in epithelial and mesenchymal breast cancer tumor cell types which it was originally found.

The breast cancer mouse models created in our lab consist of two different types, "double negative" and "triple negative". The "double negative" mice have Rb and p53 knockouts, while the "triple negative" mice have mutations of BRAC1, Rb and p53. All of the genes that have been knocked out of the mice are tumor suppressor genes, thus they readily develop breast cancer tumors. **Figure-6** shows micrographs of example tumors from the double knockout mice.

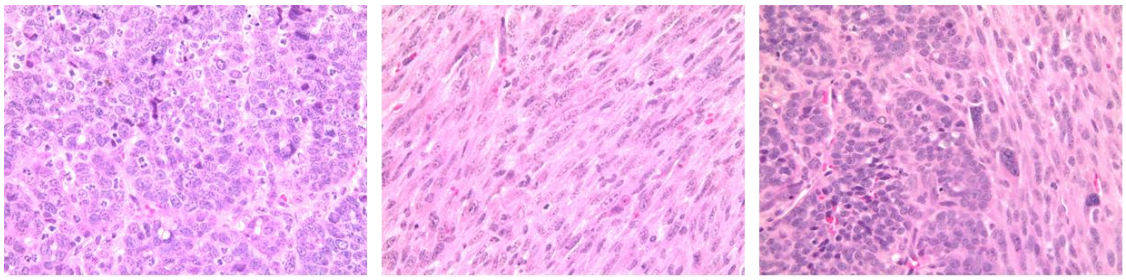

Figure-6: Micrographs of Tumors from Double Knockout Mice. Left panel shows adenocarcinomas, middle panel shows spindloid tumor, and the right panel shows both tumors side by side. 40X magnification.

The miR-30 family of miRNA has been located in two tumor cell types in the mouse models, particularly in epithelial solid tumor types (adenocarcinomas) and mesenchymal spindloid tumors. The mouse models develop both types of tumors in breast tissue. In a past study, miR-30c_2 was found to be a member of a common expression signature characterized in several solid cancers (Calin and Croce, 2006). In our "triple negative" mice, *high* cellular levels of expression of miR-30a-e were found in both tumor types (unpublished work Simin 2005). However, in the "double negative" mice the miR-30 family was found in *low* levels of expression in both tumor types. Thus, our preliminary data indicate miR-30 family members can be up or down regulated depending on the specific miRNA in breast cancer progression.

Target Scan Software

Mir "seed regions" are 2-8 bp long domains of a miR that bind by complementary base-pairing to the 3'UTR of the target mRNA to induce its dormancy or degradation. Target Scan is an algorithm that helps identify seed regions, and predicts miRNA gene targets using the mer system which matches the seed regions of particular miRNAs to gene sequences. When testing a variety of miRs, the more highly conserved

a nucleotide sequence at a particular domain, the more likely that domain is its seed sequence that binds to that gene to downregulate it.

The Simin lab used Target Scan to find the seed regions and target genes of the miR-30 family. The search indicated that RUNX2 and metadherin mRNAs likely bind this family. RUNX2 and metadherin are involved in cell proliferation and metastasis, thus one working model for the miR-30's is their levels likely *diminish* in tumors (in agreement with Iorio 2005 who showed miR-30s are all down-regulated in both estrogen receptor-negative and progesterone receptor–negative tumors). Low miR-30 levels in tumors would increase the cellular levels of RUNX2 and metadherin to stimulate cell proliferation. Its strong expression in normal tissue could act to downregulate the RUNX2 and metadherin proliferation stimulators.

Creation of the tet-Regulated miR Expression System

The purpose of this project was to create an inducible expression system to investigate the effect of overexpressing specific microRNAs in a cell. Since the effects of over-expression could be detrimental to a cell, an inducible system was devised to control the time at which miR expression is induced. In addition, an inducible system would allow the effects of various doses of miR to be determined, or to determine the effects of long term miR-30 expression on a cell.

In a previous study using a miR-30a backbone, previous investigators were able to achieve a tet-regulated system from a U6 promoter (Zhou et al., 2008). The transcriptional activity of Tet-regulatable U6 promoter was tightly regulated using

Tetracycline. Repeats of a Tetracycline Operator sequence were introduced within the promoter regions and also between U6 promoter and miRNA gene (Zhou et al., 2008).

Thus for our study, the vector chosen to be apart of the tet-regulation system was pTRE-Tight-DsRed2 (**Figure-7**). This plasmid has a tet-responsive element (TRE) that activates expression of any gene inserted downstream when a high enough concentration of a tetracycline transactivator (tTA) protein is present (**Figure 8**). The tTA-responsive plasmid has a built-in reporter, Tight-DsRed2 (TRITC), which glows red under fluorescence when taken up by cells. The expression of this reporter is also under control of the TRE element, so it is also induced by tTA.

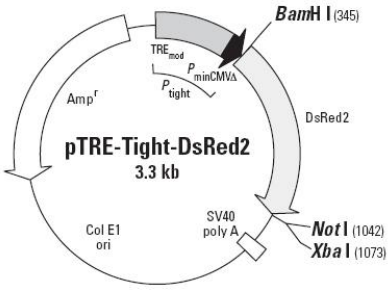

Figure-7: Diagram of the CloneTech tTA-Responsive Plasmid.

The Tet-responsive plasmid is used in tandem with a second plasmid encoding a tetracycline transactivator (tTA) (**Figure-8**). When plasmid pTet-On is used, the tTA is expressed only in the presence of Tet or Dox. In the presence of Tet or Dox, tTA is synthesized, which binds the TRE element of the reporter plasmid to induce expression of the miR and DsRed2. When plasmid pTet-Off is used, tTA is constitutively expressed, which causes constitutive expression of the miR and the DsRed2.

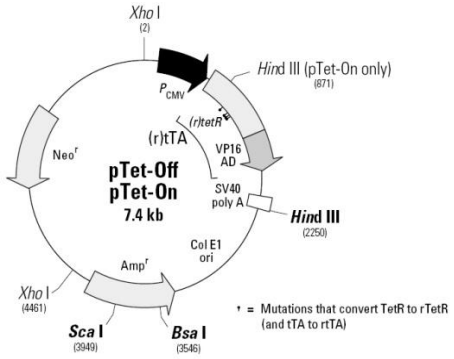

Figure-8: Diagram of the tTA Plasmids pTet-On and pTet-Off by ClonTech.

Using this dual plasmid system allows control of the miR synthesis, which should be a superior approach to previous studies in which the miR RNA was directly transfected, since the oligos in the latter technique can be rapidly degraded.

PROJECT PURPOSE

The purpose of this project was to create a tet-regulated system to control expression of miR-30 microRNAs in cancer cells, which could eventually be used to gain insight of the functions of microRNAs in their role in breast cancer. In order to test this, four genes encoding miR-30c_1, c_2, a, and d, were cloned from MDA-213 DNA using PCR. The DNA for miR-30c_1 was then cloned into a TRE-responsive expression vector, then transfected into a human embryonic kidney cell line (HEK-293) and a derived breast cancer cell line (MDA-213). Plasmid pTet-On encoding an active tetracycline transactivator (tTA) was also transfected into the cells, to interact with (stimulate) the tet-responsive element of the plasmid. Once transfected, doxycycline was added to the cells to induce expression of the tTA, which upregulates miR-30c_1 expression within in the cells. Once the cells are successfully transfected, the DsRed2 reporter will fluoresce red in the presence of Tet or Dox as a measure of transfection efficiency. Real Time Reverse Transcriptase PCR will then be performed to quantitate the amount of miR-30c_1 RNA present in the cells.

METHODS

Cloning

PCR Amplification of MiR-30c$_1$

miR-30c$_1$ was chosen as the insert for this experiment because of its presence in breast cancer tumor cells, and its target mRNAs were identified by the Target Scan software. This insert had to be amplified for use in further experiments. The DNA encoding mir-30c$_1$ was amplified by PCR from MDA-231 genomic DNA in the following reaction: 1.6 µl dNTPs, 0.4 µl of both forward and reverse primers, 1.0 µl MDA-231 genomic DNA, 0.2 µl Taq polymerase, 4 µl buffer, and 12.4 µl water. This solution was then placed in the thermocycler and 8 reaction tubes were run for 8 cycles.

Restriction Digestion

In order to insert the miR-30c$_1$ PCR amplicon into pTRE-Tight-DsRed2, restriction enzymes SmaI and NheI were used which create blunt ends. 1.0 µl of miR-30c$_1$ DNA along with 1.0 µl each of the restriction enzymes, 5 µl NEB Buffer 4 and 41.5 µl water was incubated at 25°C (room temperature) for half an hour. Then this was removed and placed in a 37°C water bath for one hour.

Another restriction digest was performed in conjunction with the aforementioned digest to test whether the miR-30c$_1$ insert was correct. In this case, restriction enzymes XbaI and NheI were used because of their known cut sites on the plasmid. The miR-30c$_1$ amplicon DNA was combined with the restriction enzymes, BSA, and NEB Buffer, and

placed into a 37°C water bath for one hour. This was then analyzed on a1.0% agarose gel to verify the correct size (500 bp).

Ligation

Once the insert was verified to be miR-30c$_1$, the insert was ligated to the cut pTRE plasmid. 5 µl of the miR-30c$_1$ DNA was combined with 5 µl of pTRE-Tight-DsRed2 that had been cut during the double digest, 10 µl of the 2x quick ligation buffer and 1 µl of t4 quick DNA ligase. This was incubated at room temp for ½ hour.

Transformation

For the transformation, DH5α *E. coli* cells were used. LB+Ampicillin plates were placed in the 37°C incubator to be warmed for the cell spreading. Then 1.0 µl of ligation reaction was placed into 50 µl DH5α cells and 250 µl S.O.C. media. This solution was placed into snap-cap tubes and then was transferred to a floor shaker at 37°C for one hour. Afterwards the tubes were removed; the LB+Ampillicin plates were taken from the incubator and placed on the lab bench in a sterile area where the cells could be spread on the plates. Varying concentrations were plated: 50 µl, 100 µl, 150 µl and 200 µl to find the best colony growth. These plates were then placed in the 37°C incubator for 24 hours.

Screening of the miR-30c$_1$-pTRE-Tight-DsRed2 Plasmid

Colonies were picked from the 150 µ1 and 100 µl plates since they had many colonies to choose from, and the plate was not completely covered in colonies and satellite colonies. Eight colonies were picked, and each was combined with 20 µl buffer,

6 µl $MgCl_2$, 32 µl dNTPs, 4 µl of both forward and reverse primer, 5 µl, 1 µl Taq polymerase and 117 µl dH_2O (for 8 reactions).

The positive control for this screening was the MDA-231 genomic DNA which was also combined with the aforementioned reagents. The negative control was the empty vector (without the ligated miR-$30c_1$) and it was also combined with the PCR mixture. These solutions were transferred to PCR tubes and then placed in the thermocycler for one hour. After the thermocycler completed its 8 cycles, a 1.0% agarose gel was made containing ethidium bromide and the samples were loaded into the gel. After the bands ran about ¾ of the way down the gel, the gel was photographed using UV light. The presence of miR-30$_{1c}$ amplicons from the cloned plasmids indicated positives. Positive colonies identified from the screen were frozen as 50% glycerol stocks at -80ºC.

Plasmid Maxi Preps for Transfections

In order to prepare sufficient clean quantities of the cloned plasmid miR-$30c_1$-pTRE-Tight-DsRed2 for transfection experiments, maxi preps were prepared. Two LB+Amp plates were placed in the incubator and a tube containing a positive culture screened in the previous section was thawed. After the plates were warmed, they were moved to a sterile area, and streaked with 50 µl or 150 µl of culture. Plates were returned to the 37°C incubator for 24 hours.

After the incubation, two fresh individual colonies were picked from the 50 µl plate and placed in 5 ml S.O.C. media in a snap-cap tube, and then placed in the floor shaker for 8 hours at 37°C. LB media with ampicillin was made for the next step of the

growing up of the plasmid. After this incubation period, the tubes were removed and the contents were each placed in 500 ml of LB media in Erlenmeyer flasks and returned to the 37 °C floor shaker for 24 hours. After this incubation, the media was split into 250 ml portions, spun down in a centrifuge, and the supernatant was removed, leaving the cell pellet. Then the two pellets from the same colony were recombined.

After the recombination of pellets, a maxi prep was performed using the Quiagen Maxi Prep Kit to extract the DNA grown up in the cells. These samples were placed in the spectrometer to find the concentration of the plasmid. The highest concentration of the two samples was 120 µg/ml.

Transient Transfections

Growth of the Cell Lines

The cell lines that were used the transfections were human embryonic kidney cell line (HEK-293) and human-derived breast cancer cell line (MDA-231). The HEK-293 cell line was chosen because it is easy to grow and manage. The first transfection is of the HEK-293 cells, the second transfection is of the MDA-231 cells.

Transfection of the HEK-293 Cell Line

After the successful Maxi Prep, the HEK-293 cells were split for the transfection. The cells were count using trypan blue and a hemacytometer, and the needed amount per well of the 6-well plate was 3.5×10^5 cells. The amount of volume was calculated based on the amount of cells available, 3.75×10^6 and the needed volume of cells and 2 ml

media per well were placed in each well of the transfection plates and these were grown overnight.

In order to do the transfection, Fugene, a chemical that allows the plasmid to be taken in by the cells was used to successfully deliver the plasmid into our cell line. Rev-tet off was also used to have miR-30c_1 constitutively expressed in the cells since at this point we did not know what would happen to the cells if high levels of miR-30c_1 were present. The control for this transfection was one well containing only cells and not the transfection agents. The other wells had varying concentrations of the miR-30c_1: 0.01 µg, 0.5 µg, 1.0 µg, 1.5 µg and 2.0 µg. After the transfection, these plates were placed into a 37°C incubator for 48 hours.

qRT-PCR of the HEK-293 Transfection

Following transfection, total cellular RNA was isolated from the cells by using the PARIS kit, provided by Ambion Systems. In order to quanitate the cellular levels of miR-30c_1 RNA in the cells, a qRT-PCR was performed. The primers used in this assay were TaqMan. For each 15 µl reaction, the ratio of RT master mix to total RNA is 7 µl to 5 µl. We used 10 ng of cellular RNA diluted to 2ng/µl. Thus 5 µl was used of RNA, 3 µl of primer and 7 µl RT master mix. After everything was combined, the solution was transferred to PCR tubes and placed in the thermocycler for 65 min.

Once the cDNA was made, the RT product, primer, master mix, and water were calculated for 10 µl reactions and for each concentration miR-30c_1 performed in triplicate. Thus a 384-well plate was needed for the loading control, U6, miR-30c_1 and miR-30e (miR-30e was another test), a total of 99 samples. Table I shows the calculations and how the 384-well plate was loaded.

Reagents	96-well plate (20 µl per reaction)	384-well plate (10 µl per reaction)
Primer	1 µl	0.5 µl
RT Product	1.33 µl	0.67 µl
Master Mix	10 µl	5 µl
Nuclease Free Water	7.67 µl	3.83 µl

Table 1: Reagent Calculations for RT-PCR. This RT-PCR amplification was performed for 120 reactions

Transfection of the MDA-231 Cell Line

For this transfection, pTet-On was used to control the cellular levels of miRNA expression within the cells and to optimize the tet-responsive element in the plasmid. The pTet-On had been ligated with the pTRE-Tight-DsRed2 plasmid and was combined with DH5α cells for the transformation. Two LB+Amp plates were placed in the 37°C incubator. After the plates were warmed, they were streaked with 50 µl or 150 µl of cell suspension. These were returned to the 37°C incubator for 24 hours. After the incubation, two fresh individual colonies were picked from the 50 µl plate and used to prepare a maxi-prep as described previously, except 500 ml cultures were used. The highest concentration from the four samples was 450 µg/ml.

After the successful Maxi Prep, the MDA-231 cells were split for the transfection. The cells were counted using trypan blue and a hemacytometer. The needed amount of cells for a 6-well plate was 3.5×10^5 cells per well, so the volume was calculated based on the amount of cells available, 3.67×10^5 and 1.97×10^5, which were taken from the total collection of MDA-231 plates. The required volume of cells and 2 ml of media per well were placed in each well of the transfection plates and these were grown overnight.

One of the six-well plates was dedicated to controls, and the other plate was the experimental plate.

As described before, Fugene was used to deliver the plasmid into the cell line. The controls for this experiment were a cells-only well, an empty vector that did not contain miR-30c_1 well, fugene-only well, and the plasmid without PTet-On. The other wells had varying concentrations of the pTet-On-pTRE-Tight-DsRed2 plasmid: 0.5 µg, 1.0 µg, 1.5 µg, and 3.0 µg. After the transfection, these plates were placed into a 37°C incubator for 48 hours.

To induce the TRE-plasmids with Dox, the highest recommended dose of doxycycline was added to each of the wells, which was 1.5 µl per well. After the induction with doxycycline, the cells were incubated for one hour then observed under the fluorescent microscope for the presence of the red DsRed2 reporter.

RESULTS

The purpose of this project was to create a tet-regulated system for the expression of different types of miR-30 RNAs (miR-30c_1, c_2, a, and d) in HEK-293 and MDA-231 cells. The miR-30 family in general was chosen for investigation because their seed sequences are predicted to bind mRNAs for RUNX2 and metadherin, two metastasis inducing genes. The family was also selected because of its presence in the lab's double and triple negative mouse breast cancer models, and its presence in spindloid and adenocarcinoma tumors. In particular, miR-30c_1 was chosen for over-expression due to its low levels in spindloid tumors.

The first step in the process of making the tet-regulated expression system was to select a plasmid that could be taken up by the cells and regulate the amount of miRNA present for future assays. The plasmid chosen was ClonTech's pTRE-Tight-DsRed2, because of its built-in reporter gene, TRITC, which encodes the red fluorescing protein DsRed2, and its tet-responsive element (TRE) promoter, which allows for tet-regulation. The process also uses a sister plasmid that expresses the tetracycline transactivator (tTA) in the presence of tetracycline or doxycycline. The tTA binds the TRE element of the first plasmid to switch on expression of both the miR-30c_1 and the DsRed2

DNA encoding four miR-30's (c_1, c_2, a, d) were cloned from MDA-231 genomic DNA by PCR. The amplicons were cut with restriction enzymes to verify they represented miR-30 DNA. To clone the DNAs into the TRE expression vector, the DNAs were cut with enzymes to produce blunt ends for cloning. The blunt ended DNAs were ligated into the TRE plasmid, and positives were screened by restriction digestion to

remove insert (**Figure-9**). The data indicated the successful cloning of all four miR-30 DNAs.

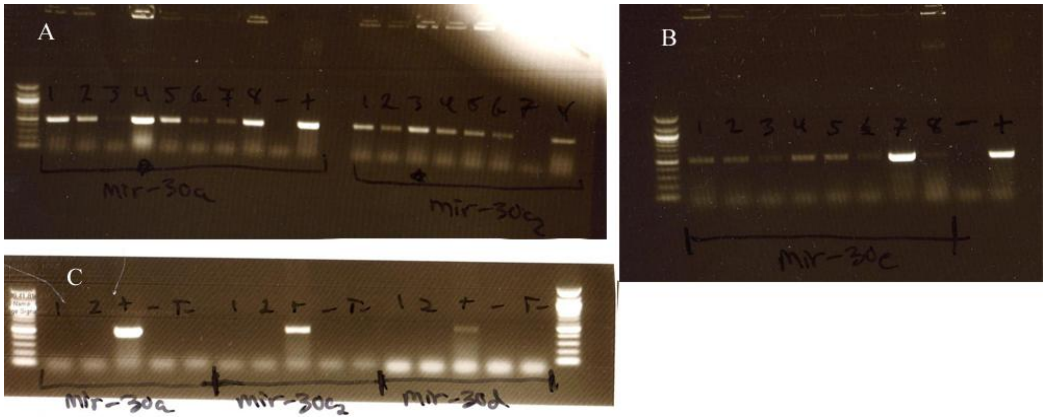

Figure-9: PCR Screening of Positive TRE Plasmids Encoding Four miR-30 RNAs. These are the gel electrophoresis results of transformation of the miR-30 of interest and pTRE-Tight-DsRed2 into DH5α cells. Panel A represents positive results for miR-30a and miR-30c_2. Panel B represents positive results for miR-30c_1. Panel C represents positive results for miR-30a, miR-30c_2 and miR-30d. The positive control for all of these gels was the MDA-231 genomic DNA as indicated by the plus sign. The negative control for all of the gels is the vector without insert.

Two cell lines were chosen for transfection, human embryonic kidney cells (HEK-293) and human derived breast cancer cells (MDA-231). HEK-293 cells were used first to test whether the clones could increase the detectable amount of miR expression. The plasmid encoding miR-30c_1 was chosen for transfection, at various masses of plasmid (**Figure-10**).

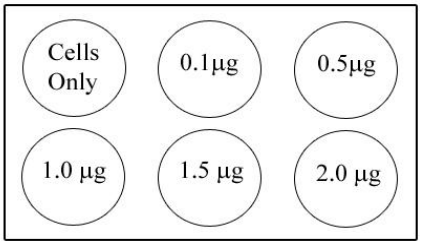

Figure-10: Diagram of the 6-Well Plate Setup for the miR-30c_1 Transfection Experiment. This is the 6-well transfection plate with the varying concentrations of miR-30c_1 plasmid. Each well contained 2 ml of media, and a ratio of 2:3 DNA to Fugene per well. They also all contained the same amount of Rev-Tet Off to have the cells constitutively express miR-30c_1.

Table II shows the amounts of each reagent used for the transfections. Rev-Tet-Off is a plasmid encoding an inactive tTA and it was used to temporarily suppress expression of the miR since its effects on cell growth are unknown and could be detrimental to the cell.

Reagent	Well 2	Well 3	Well 4	Well 5	Well 6
Rev-Tet off	1.26 µl	1.26 µl	1.26 µl	1.26 µl	1.26 µl
MiR-30 c1	0.1 µg	0.5 µg	1.0 µg	1.5 µg	2.0 µg
Fugene	2.4 µl	3 µl	3.75 µl	4.5 µl	5.25 µl
Media	100 µl	100 µl	100 µl	100 µl	100 µl

Table II: Reagents Added for Transfection. This is the calculated reagents per well. The concentration of Rev-Tet off is 1.19 µg/µl and the concentration of miR-30c_1 is 120 µg/ml.

After 48 hours of incubation, the plates were examined under the fluorescent microscope to see if the cells had taken up the plasmid, as indicated by the red fluorescing reporter DsRed2 (TRITC) (**Figure-11**). The data indicate that approximately 30% of the cells had taken up the TRE miR-30c_1 plasmid in the transfection (note that the red fluorescent signal is white in the figure).

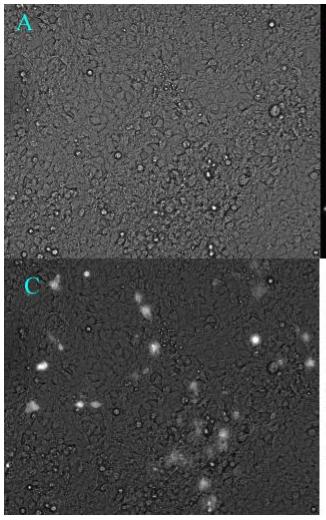

Figure 3: Fluorescent microscopy of the 1.5µg concentraion of mir-30c1 and plasmid. Panel A represents the cells in light microscopy. Panel B represents the cells in fluorescent microscopy, the glowing cells indicating the successful transfection of the plasmid. Panel C represents the combination of fluorescent microscopy on top of the light microscopy to show which cells are glowing.

Figure-11: Fluorescence Microscopy of HEK-293 Cells Transfected with TRE Plasmid Encoding miR-30c_1. The data indicate that approximately 30% of the cells had taken up plasmid.

To determine whether the transfected cells increased their levels of miR-30c_1 RNA, total RNA was isolated from the cells, and qRT-PCR was performed using miR-30 primers (**Figure-12**). Due to the large number of samples, a 384-well plate was used in the real-time portion of the PCR. The loading control was U6, a small RNA constitutively expressed in the cell; it is quanitated to ensure the same amount of RNA is in each sample. The cellular levels of expression were normalized relative to the amount of U6 in each sample. The data indicate an increase of miR-30c_1 RNA in cells transfected with plasmid, especially at the 1.5 µg dose. A 0.1 µg sample is missing because it was contaminated during the transfer to create a triplicate. The expression levels diminished at the highest dose tested.

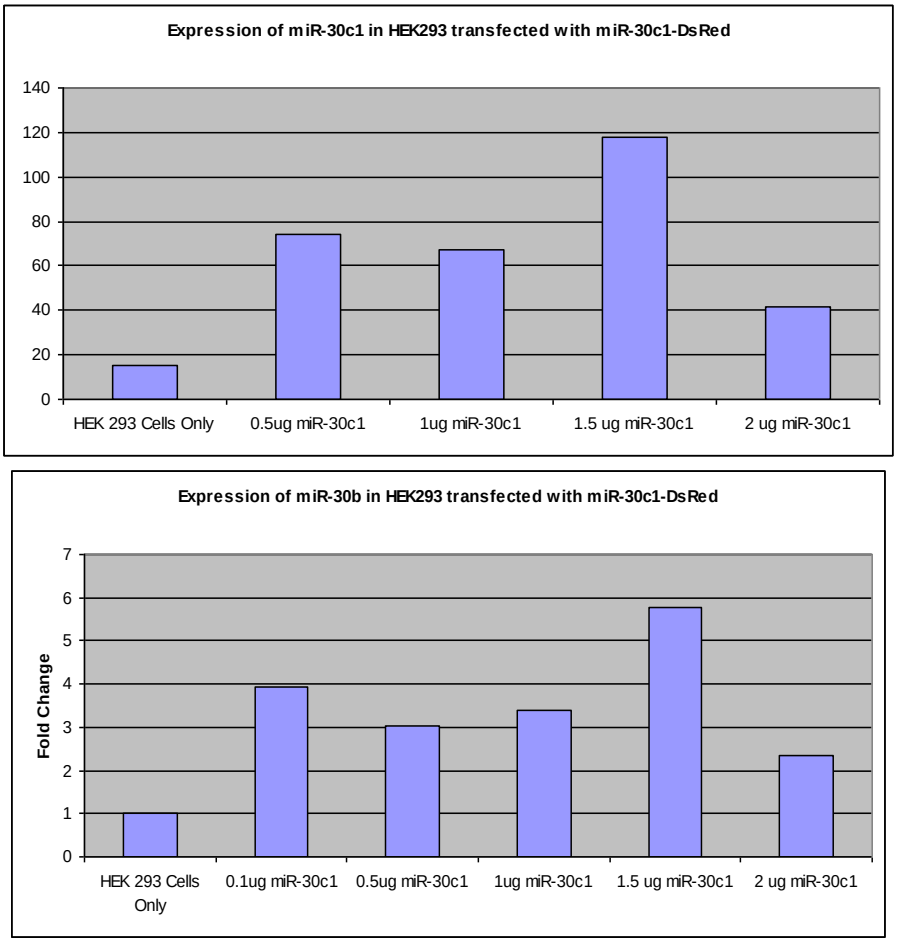

Figure-12: qRT-PCR Assay of the Levels of miR-30c$_1$ in Transfected Hek-293 C. The upper panel denotes raw OD data, while the lower panel denotes the data normalized relative to untransfected cells. As seen in this graph, detectable levels of miR-30c$_1$ increased relative to non-transfected cells, especially in the 1.5 µg DNA sample. The lowering of expression observed in the 2.0 µg concentration could attribute to that the cells can only withstand a certain amount of miR-30 and an overloading of the system may have occurred. All miR-30c$_1$ levels were normalized relative to U6 small RNA.

In order to test whether over-expression of miR-30c$_1$ induced the expression of another miR-30 RNA, primers to miR-30b were used in qRT-PCR (**Figure-13**). The data indicate surprisingly that the levels of miR-30b did increase, and were maximal in the cells maximally expressing miR-30c$_1$.

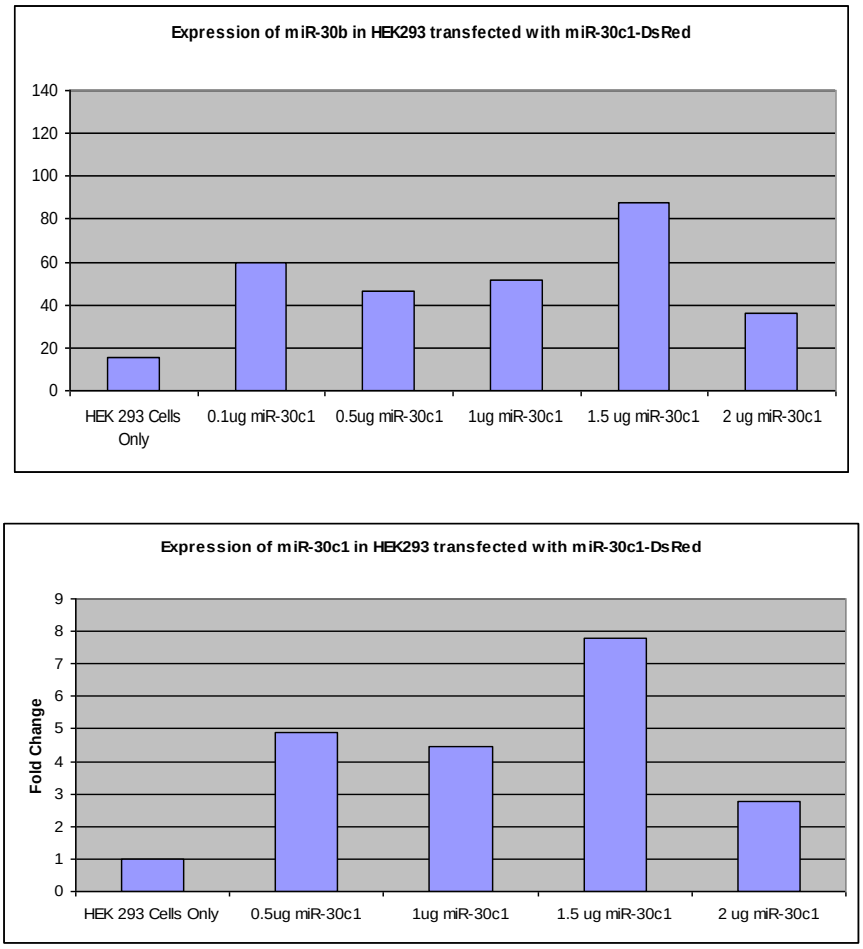

Figure-13: qRT-PCR Analysis of the Cellular Levels of miR-30b in HEK-293 Cells Overexpressing miR-30c$_1$. This graph represents the cellular levels of expression of miR-30b. The upper panel denotes the raw OD data, while the lower panel denotes the data normalized relative to untransfected cells. Note that its expression is highest at the same 1.5 µg plasmid dose that gave the maximal miR-30c$_1$ expression.

The next test was to determine whether the miR-30c$_1$ expression could be controlled within the cells using the tet-responsive element of the plasmid. By co-transfecting the pTet-On plasmid, encoding a Tet or Dox activatable tTA which would bind the TRE of the miR plasmid inducing its expression, a system was created for an inducible miR system. The plate setup for the transfection consisted of one control plate (**Figure-14**) and one experimental plate (**Figure-15**).

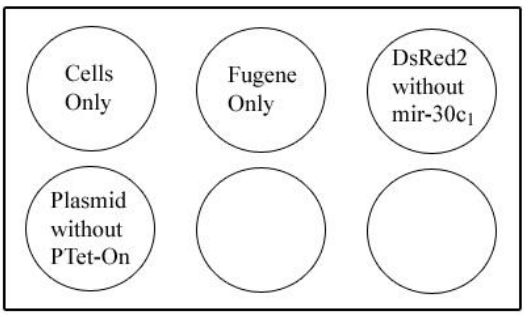

Figure-14: Diagram of the Control 6-well Transfection Plate. The amount of Fugene used in the fugene well was the maximum recommended amount which was 6.75 µl. The amount of the plasmid without the ligated miR-30 c_1 is 1.5 µg. The amount of plasmid without PTet-On is 3.0 µg which was the maximum used in our experimental plate. Each well contained 2 ml of media, and a ratio of 2:3 DNA to Fugene per well. The last two wells were cells only.

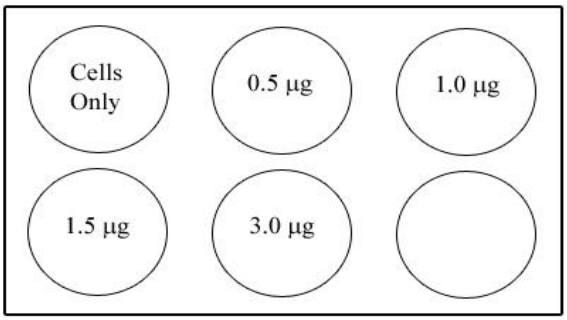

Figure 15: Diagram of the Experimental 6-well Transfection Plate. The varying concentrations represent the concentrations of miR-30c_1-DsRed2 plasmid. Each well contained 2 ml of media, and a ratio of 2:3 DNA to Fugene per well. The last well was cells only.

E. coli cells containing plasmid pTet-On were streaked on plates in 50 µl and 150 µl concentrations, then fresh individual colonies were picked, grown in SOC media, then in LB + Ampicillin media. The Qiagen Maxi Prep procedure was performed to extract and purify the plasmid DNA, and the final concentration was found to be 450 µg/ml. 3.5 x 10^5 MDA-231 cells were seeded per well, and the transfection was set-up as described in **Table III**. 1.5 µg of pTet-On (3.33 µl) was added per well.

Reagent	Well 1	Well 2	Well 3	Well 4
mir-30c1-DsRed2	0.5 µg	1.0 µg	1.5 µg	3.0 µg
Fugene	3 µl	3.75 µl	4.5 µl	6.75 µl
PTet-On	3.33 µl	3.33 µl	3.33 µl	3.33 µl
Media	100 µl	100 µl	100 µl	100 µl

Table III: Transfection Setup for the Double Transfection pTRE-miR-30c$_1$ + pTet-On Experiment. Shown are the calculated reagents per well. The concentration of PTet-On is 0.45 µg/µl, and the concentration of miR-30c$_1$ is 120 µg/ml.

After 24 hours incubation, doxycycline was added to all of the experimental wells to activate the tet-responsive element of the plasmid. After one hour, both plates were observed for expression. Unfortunately none of the wells showed any expression, which would have been indicated by red fluorescence. The pictures of the fluorescence were just black screens. We did not proceed with the qRT-PCR because of these negative results. This experiment was not repeated in HEK-293 cells which had shown the previous successful fluorescence data.

DISCUSSION

The data from this project show that a system to express miR-30c$_1$ was successfully constructed. Fluorescence microscopy on transfected HEK-293 cells indicated strong expression of the marker protein DsRed2, and qRT-PCR on transfected HEK-293 RNA indicated strong expression of miR-30c$_1$. Thus it is possible to make a system in which miR-30 plasmid can be transported into cells. This procedure may not be as effective as direct transfection of the synthetic oligos, but this comparison was not tested in this project. However plasmid transfection is a cost-effective tool and will be optimized in the future to achieve higher levels of expression. Also when the assay becomes optimized eventually a stable cell line can be created to produce a system that continuously expresses miR-30c$_1$, which is not possible with synthetic oligos.

The main problems with this project were the initial cloning, and the transfection of the MDA-231 cell line. The issue with cloning was that an initial vector (FIV plasmid), was not efficiently transformed into DH5α cells. The screening repeatedly came out negative, and when there was a slight positive result it did not pan out on subsequent screening. It was thought that the *E. coli* cells were being killed off by the plasmid. So a new plasmid was chosen, the pTRE-Tight-DsRed2 which included a built in reporter and had the tet-responsive element. Cloning was successful with this plasmid as was the primary transfection in HEK-293 cells.

But when performing the MDA-231 transfection, we only tested one amount of doxycycline, and it was unclear how much pTet-On should have been used per well since it was unknown how much pTet-On could be detrimental to the MDA-231 cells. There

may have been too much PTet-On and doxycycline in the system and it killed off the cells, or there may have been too little, and there were not sufficient quantities of plasmid to fluoresce. Concentrations that were used in this transfection were based off of the HEK-293 transfection since it yielded positive results at certain concentrations of miR-30c_1. In addition, HEK-293 cells are traditionally easy to transfect, so it is possible that the MDA-231 cells are simply more resistant to transfections. Time did not allow us to repeat the tet experiment in HEK-293 cells.

For future experiments, we want to optimize the transfection and find out how much doxycycline and PTet-On is sufficient for the plasmid to be expressed. More concentration tests of the plasmid could be performed in conjunction with exploration on the amount of doxycycline. This could be done with more transfections of the MDA-231 cell line (and also the easier to transfect HEK-293 line) and then analyzed with qRT-PCR.

Another future experiment would be to create stable transfectants for constitutively producing miR-30c_1 to determine its long term effects on the cell. These experiments are not possible when directly transfecting oligos because the oligos are eventually degraded. With the stable cell line, time-point assays could be performed since the cells could replicate and retain the plasmid in their genome. Such assays that could be done to gain insight of the functions of miR-30's, since it is unknown how miR-30's play a role in tumor oncogenesis, and assays that could be performed are proliferation and invasive assays to see how they might act as an oncogene or tumor suppressor.

Lastly we could use qRT-PCR and western blots to investigate whether RUNX2 or metadherin mRNAs or proteins, respectively, are downregulated in cells overexpressing miR-30c_1. These two target genes have been predicted to bind the seed sequences of miR-30 family members using Target Scan software.

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