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Phan H. Nguyen April 2, 2012

# Development of a Novel MicroRNA Screening System by Identifying the Candidate MicroRNAs that Target Oct4

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Abstract

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MicroRNAs (miRNAs) are posttranscriptional modulators of gene expression that act by directly targeting its 3' untranslated region (3'UTR) and play an important role in many developmental processes. Aberrant regulation and altered expression of specific miRNA genes contribute to the initiation and progression of cancer stem cells (CSC). To date, more than 1000 human miRNAs have been documented, but the functions of most of them remain elusive. The main purpose of this study is to set-up an efficient miRNA screening system of a total of 931 human miRNAs by combining standard transfection protocols with high throughput screening (HTS) and high content analysis (HCA) equipments from the Emory Chemical Biology Discovery Center (ECBDC). Oct4 was chosen as the initial target gene since it is an essential transcription factor in embryonic stem cell (ESC), as well as cancer stem cell (CSC), development and it is a small gene with a short 3'UTR, which allowed for easy initial set-up and modifications of this novel screening system. The system uses fluorescence signals, which correlate to the targeting effects of miRNAs on target genes, to narrow down the large miRNA library to facilitate focused studies with a more manageable miRNA sample size. The initial screening identified miR-4310 and miR-1253 as candidates that target Oct4-3'UTR. Western blotting results verified the target relationship of Oct4 and the miRNAs and further confirmations of the findings by luciferase assays and miRNA dose response curve to pDsRed2-Oct4-3'UTR will be followed. The identification of these miRNA candidates against Oct4 from a library of 931 miRNAs revealed that the new screening approach is promising and efficient, although improvements are still necessary to strengthen the system. Nonetheless, with the establishment of this novel and automatic miRNA screening system, the 3'UTR of any desired gene can be screened for its miRNA regulators within a reasonable time frame, while also reducing the extensive labor, human error, and supply cost associated with manual screenings.

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Sincerely,
Phan Nguyen

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**Abstract**

MicroRNAs (miRNAs) are posttranscriptional modulators of gene expression that act by directly targeting its 3' untranslated region (3'UTR) and play an important role in many developmental processes. Aberrant regulation and altered expression of specific miRNA genes contribute to the initiation and progression of cancer stem cells (CSC). To date, more than 1000 human miRNAs have been documented, but the functions of most of them remain elusive. The main purpose of this study is to set-up an efficient miRNA screening system of a total of 931 human miRNAs by combining standard transfection protocols with high throughput screening (HTS) and high content analysis (HCA) equipments from the Emory Chemical Biology Discovery Center (ECBDC). Oct4 was chosen as the initial target gene since it is an essential transcription factor in embryonic stem cell (ESC), as well as cancer stem cell (CSC), development and it is a small gene with a short 3'UTR, which allowed for easy initial set-up and modifications of this novel screening system. The system uses fluorescence signals, which correlate to the targeting effects of miRNAs on target genes, to narrow down the large miRNA library to facilitate focused studies with a more manageable miRNA sample size. The initial screening identified miR-4310 and miR-1253 as candidates that target Oct4-3'UTR. Western blotting results verified the target relationship of Oct4 and the miRNAs and further confirmations of the findings by luciferase assays and miRNA dose response curve to pDsRed2-Oct4-3'UTR will be followed. The identification of these miRNA candidates against Oct4 from a library of 931 miRNAs revealed that the new screening approach is promising and efficient, although improvements are still necessary to strengthen the system. Nonetheless, with the establishment of this novel and automatic miRNA screening system, the 3'UTR of any desired gene can be screened for its miRNA regulators within a reasonable time frame, while also reducing the extensive labor, human error, and supply cost associated with manual screenings.

## 1. Introduction

### 1.1. The importance of microRNAs (miRNAs) in gene regulation

MicroRNAs (miRNAs), in conjunction with short interfering RNAs (siRNAs), are one of two regulators that have emerged as important players in post-transcriptional regulation and messenger RNA (mRNA) decay. MiRNAs are small, non-coding single strand RNA molecules found in eukaryotic organisms. They are highly conserved and usually 18-23 nucleotides (nt) in length. MiRNA represses gene expression by base pairing with the 3' untranslated regions (3'UTR) of target mRNAs to promote mRNA degradation or a translational blockage. Since the first miRNA was discovered in (Wightman et al., 1993), over one thousand human miRNAs have been documented. miRNAs are predicted to constitute about ~1% of the known genes in humans (Bartel 2004). Most human genes are regulated by at least one miRNA (Friedman et al., 2009). One gene can be repressed by multiple miRNAs and one miRNA may repress multiple target genes, which results in the formation of a complex regulatory feedback network; therefore, the potential impact of altered miRNA levels in cellular functions and development is conceivably enormous (Bartel, 2004). Not surprisingly, miRNAs are found to be involved in almost all cell functions including cancer development (Calin & Croce, 2006).

It has been suggested that miRNAs contribute to oncogenesis because they can function as oncogenes or tumor suppressors in the multi-step process of carcinogenesis. Studies have shown that miRNAs that are located in genomic regions and amplified in cancers, such as in the *miR-155* or members of the *miR-17-92* cluster, function as oncogenes; whereas, miRNAs located in portions of chromosomes that have been deleted in cancers, such as the *miR-15a-miR-16-1* cluster, function as tumor suppressors (McManus, 2003). The abnormally expressed miRNAs found in human cancers target transcripts of essential protein-coding genes that are involved in tumorigenesis, such as the Ras oncogenes by *let-7* family members, the BCL2 anti-apoptotic gene by the *miR-15a-miR-16-1* cluster, and/or the E2F1 transcription factor by the *miR-17-92* cluster of the BCL6 anti-apoptotic gene by *mir-127* (McManus, 2003). Abnormal expression of miRNAs has been found in both solid and haematopoietic tumors by

various genome-wide techniques that include different microarray platforms and bead-based flow cytometry (Calin & Croce, 2006). These findings suggest that the main mechanism that underlies changes in the function of miRNAs in cancer cells seem to be aberrant gene expression, characterized by abnormal levels of expression of mature and/or precursor miRNA sequences compared with the corresponding normal tissues. Thus, the discovery of thousands of these non-coding miRNAs has added to the complexity of the cancer cell mechanism and development.

Biogenesis of miRNA sequences begins in the nucleus with transcription of the miRNA gene by RNA polymerase II, generating the long primary miRNA (pri-miRNA) (Bartel, 2004). Upon transcription, the pri-miRNAs undergo nuclear cleavage by the RNase III endonuclease Drosha, producing 60-70nt stem-loop precursor miRNAs (pre-miRNA) with a 5' phosphate and a 2 nt- 3' overhang (Bushati and Cohen, 2007). Subsequently, the pre-miRNA is transported across the nuclear membrane by the protein exportin 5. Dicer, another Rnase III endonuclease, cleaves the pre-miRNA in the cytoplasm approximately two helical turns away from the ends of the pre-miRNA stem loop, producing a double-stranded RNA. A helicase unwinds and cleaves the double-stranded pre-miRNA in a strand-specific direction, producing 18-23 nt mature miRNAs. Each single-stranded miRNA is incorporated into the RNA-induced signaling complex (RISC), which delivers the single-stranded miRNA to the 3' untranslated region (3'UTR) of its target mRNAs to promote mRNA degradation or translational blockage (Bushati and Cohen, 2007). As a result, the mRNA sequence cannot be translated and protein concentrations decrease.

The miRNA target sites are known to lie in the 3'UTR of mRNA in animals. This was known when miRNA lin-4 in *Caenorhabditis elegans*, was first discovered to regulate developmental timing by targeting the 3'UTR in *lin-4* (Wightman et al., 1993). Following the discovery of miRNAs in other animals, an early computational study identified most miRNAs as complementary to 3'UTR mRNA sequences (Pillai, 2005); however, despite the apparent abundance of potential miRNA/3' UTR target interactions, few animal miRNA/target pairs have been validated, to date, in a physiologically relevant

context (Didiano and Hobert, 2008). In a wide variety of developmental processes, miRNAs fine-tune or restrict cellular identities by targeting important transcription factors or key pathways (Bartel, 2004). Each miRNA could have hundreds of mRNA targets, leading to the regulation of 10% or more of the protein production for all human genes. In general, one gene can be repressed by multiple miRNAs and one miRNA may repress multiple target genes, which results in the formation of complex regulatory feedback networks (Bartel, 2004). Subsequently, due to their important roles in cell regulation, irregularities in miRNA-regulated gene expression have been found to be associated with cancers, cardiovascular disorders, and a variety of other diseases (Didiano and Hobert, 2008); therefore, the potential impact of altered miRNA levels is conceivably significant.

**1.2. The importance of developing an efficient miRNA screening system**

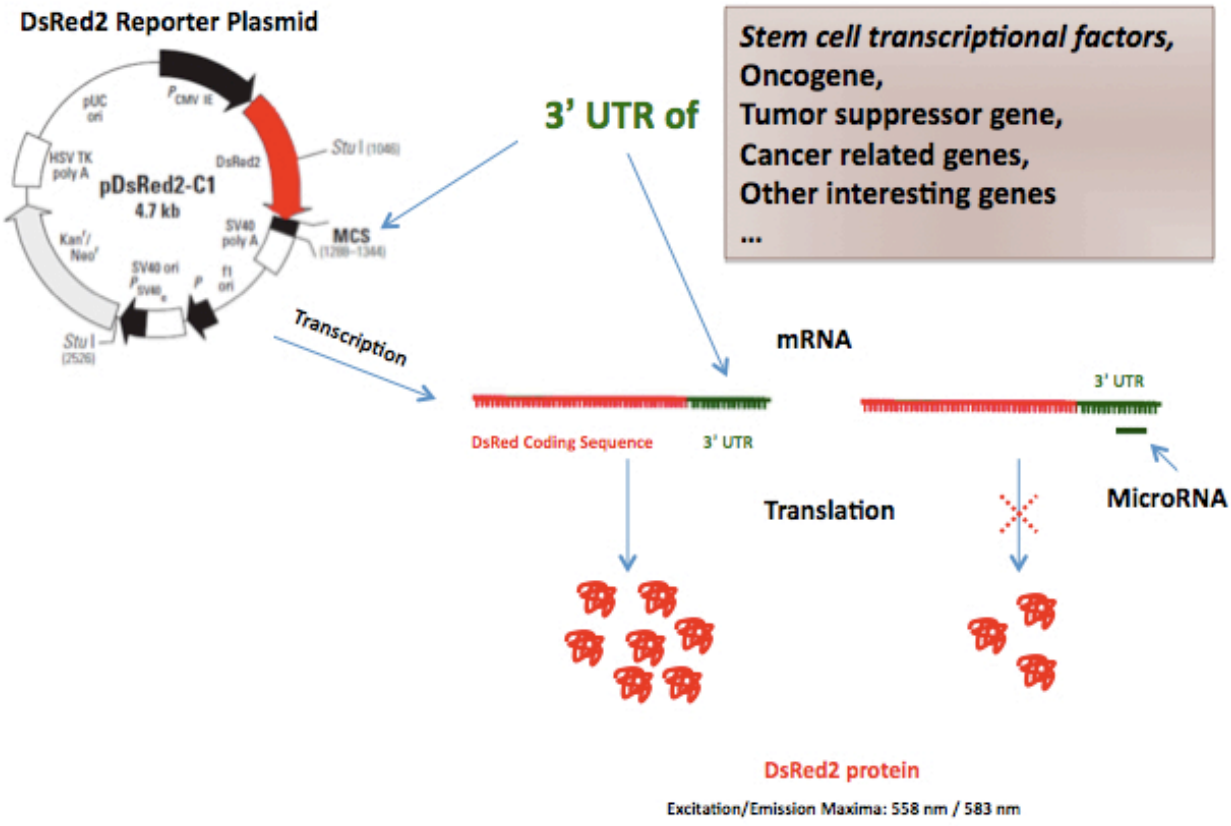
Based on miRNAs' important roles in gene regulation and cancer cell development, this study's particular interest is in the development of a reliable, sensitive, and effective screening system to individually screen a large library of 931 human miRNAs against 3'UTR of cancer related genes to facilitate focused experiments. Previously, there were several groups that used a high-throughput luciferase reporter screening system to screen for key miRNAs that could regulate p53 (a tumor suppressor gene) expression and stimulate induced pluripotent stem cells (iPSC) by manually transfecting the plasmids encoding miRNAs or miRNA mimics into cells and examining the luciferase activity (Park et al., 2009; Tian et al. 2010; Pfaff et al., 2011; Xu et al., 2009). These groups obtained some initial encouraging; however, these screenings were limited due to the manual transfections of a large pool of miRNAs (Park et al. screened 91 miRNAs, Tian et al. screened 107, Pfaff et al. screened 379 mouse miRNAs, and Xu et al. screened 466 human miRNAs), which dramatically increased the labor and supplies, generated more errors through multiple steps, and were difficult to repeat. This study aims to use a different screening method to narrow down the pool of miRNAs, identifying key miRNAs that can potentially regulate the expression of the

desired gene. From the smaller pool of miRNAs, different assays and molecular techniques can be used to further confirm the screening results.

As part of the experimental design, a pDsRed2-C1 reporter plasmid with the 3'UTR of the desired gene inserted at the multiple clone site (MCS) will be co-transfected with each miRNA transcripts into 293 FT cells, which are fast-growing, highly transfectable clonal cells derived from human embryonic kidney cells transformed with the SV40 large T antigen. Each miRNA coding sequence is attached to the 3' end of the coding sequence of a green fluorescent protein (GFP) in a plasmid construct; subsequently, the GFP expression inside the cell represents the presence of miRNA trancripts. Additionally, only the 3'UTR of the desired gene, instead of the whole gene coding sequence, is transfected since it is well known that most miRNA mechanism of gene regulation is through binding to the 3'UTR of mRNA. Screening for miRNA regulation on only the 3'UTR of desired gene, instead of the whole gene, reduced the cost of the study significantly since Oct4 gene is only found in stem cells, which are expensive to maintain on such a large scale in a laboratory setting for such a large sample-size screening. Hence, by taking advantage of the miRNA/3'UTR base pairing and upregulating the individual miRNA transcript level in vivo, the objective of this study is to develop a successful screening system that will identify the key miRNAs that can downregulate some cancer related genes by directly targeting the genes' 3'UTRs.

The transfection complex containing the miRNA expression plasmid and pDsRed2-3'UTR plasmid of the desired gene enters the cell through endocytosis, which is the process where a localized region of the cellular membrane uptakes the DNA by forming a membrane bound/intracellular vesicle (Lodish 2008). Once inside the cell, the DNA escapes the endosomal pathway, diffuses through the cytoplasm, and enters the nucleus for gene expression. Through transcription, the plasmids are transcribed into DsRed2 coding sequence encoding the 3'UTR of the desired gene and a GFP coding sequence with miRNA attached to its 3' end. Then through translation, the DsRed2 and GFP proteins are synthesized and their expressions can be viewed under a fluorescent microscope. The intensity of DsRed2 protein

expression is the key data for this experimental design. If the upregulated level of miRNA transcripts can directly target the 3'UTR of the desire gene, then translation of the DsRed2 coding sequence will be repressed, resulting in less or no synthesis of the DsRed2 proteins. If the miRNA does not directly target 3'UTR of the desire gene, then DsRed2 protein expression will be similar to the negative controls of DsRed2-3'UTR only transfection without the presence of miRNA. The downregulation of DsRed2 protein expression, by a target miRNA versus a nontargeted miRNA, can be quantified by the red fluorescent intensity readouts from DsRed2 protein expressions. The described methodology behind this experimental screening design is depicted in **Scheme 1**.

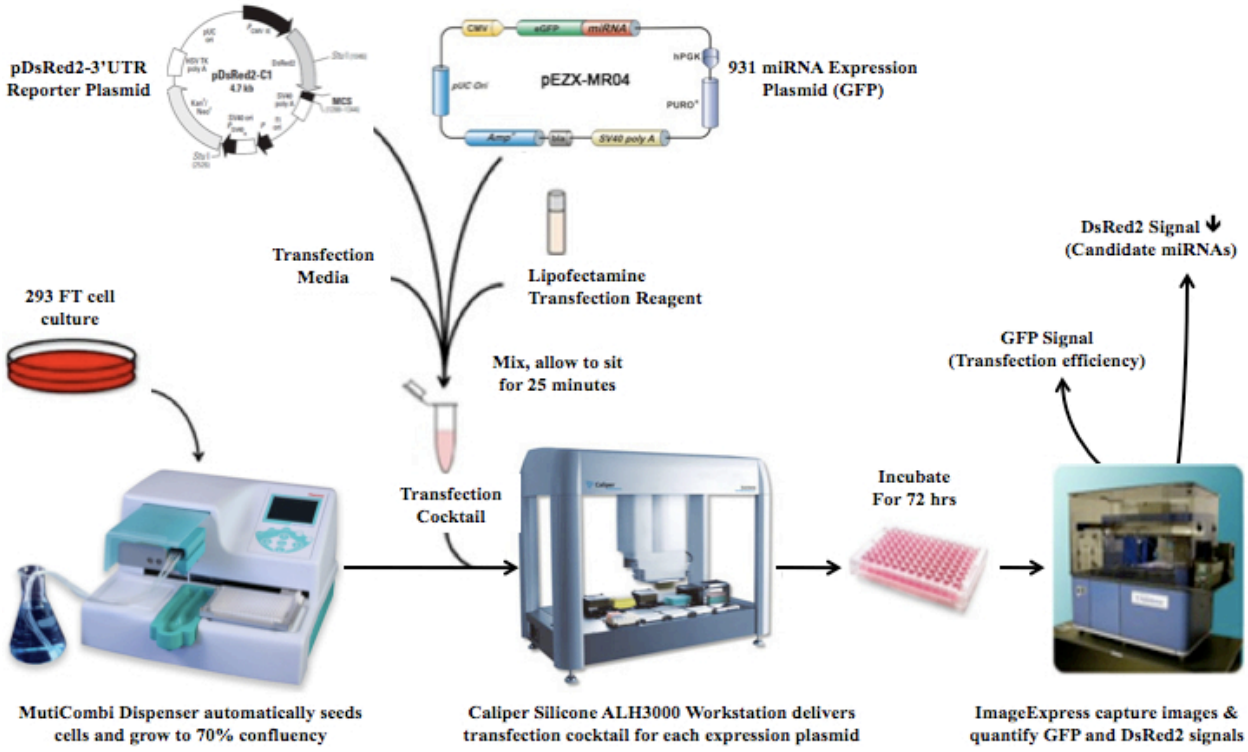



**Scheme 1. miRNA target screening design.**

As mentioned previously, various research groups performed similar miRNA screening projects against target genes, but they were all done manually, which significantly increased the labor and supplies and was subject to many human errors. In this study, by combining the robust small molecule screening equipment from the Emory Chemical Biology Discovery Center (ECBDC), a specialized application center in the National Cancer Institute Chemical Biology Consortium (NCI CBC), with fluorescence protein signal readout, a novel and more efficient screening system is developed to automatically and individually screen 931 human miRNAs, a much bigger miRNA library than seen in previous screenings. The screening facility of the ECBDC provides an attractive and technologically advanced miRNA-screening site. Through the operations with the NIH MLSCN and Emory projects, the center has built a powerful infrastructure for small molecule modulator discovery and hit optimization. The ECBDC offers state-of-the-art high throughput screening (HTS) and high-content screening (HCS) capabilities with multiple integrated robotic systems. These resources are complemented by the expertise of Dr. Haian Fu's research group in assay development and HTS. The ECBDC expertise spans from conventional biochemical/cell-based assays/screens to advanced multiplexed assays with phenotypic imaging screening. Versatile instruments for assay development include the MultiDrop combi bulk dispenser that can dispense solutions to 96, 384, and 1536 well plates in volume increments of 0.5 ul up to the capacity of the wells in each plate. Molecular Devices ImageExpress Automated cell imaging and analysis system allows for high content analysis (HCA) of 96, 384, and 1536-well plate formats. The facility also has robotic systems for high throughput dispensing, which includes the Caliper Sciclone ALH3000 Workstation system with a central vertical robot integrated with washers and dispensers equipped with pin tools for low-volume (nl) transfers. A strong informaticist and database management team supports the ECBDC. Data can also be analyzed, viewed, exported, and imported using a variety of existing tools available in this screening highly innovative facility.

Essentially, instead of performing the co-transfections of 931 miRNAs and DsRed2-3'UTR of the desired gene manually, this newly developed screening system utilizes HTS equipment and HCA from ECBDC to perform the transfections and quantify the fluorescent protein expression intensities, reducing

the labor, supplies, and time of the screening while also efficiently providing fast, processed data for analysis. **Scheme 2** provides a visual presentation of how standard mammalian transfection protocols and ECBDC's screening equipment are incorporated into this novel screening system. First, the transfection cocktails containing the pDsRed2-3'UTR reporter plasmid, individual miRNA expression plasmid, and lipofectamine transfection reagent are generated for all 931 human miRNAs. The Caliper Sciclone ALH3000 Workstation dispenses the transfection cocktails to 70% confluent 293FT cells in 96-well plates, which were seeded 24 hours before the transfections by the Multidrop combi dispenser. The transfected cells are incubated for 72 hours and then the plates are read by a Molecular Devices ImageExpress automated cell imaging and analysis system. This system captures images of the transfected wells at three wavelengths of DAPI, GFP, and DsRed2. The DAPI staining images detect the presence of live cells, while GFP signal represents the transfection efficiency of miRNA transcripts, and DsRed2 signal represents the level of DsRed2 protein expression in response to the presence or absence of miRNA downregulation on the 3'UTR of the desired gene.




**Scheme 2. Experimental design that combines transfection protocols and ECBDC screening equipment.**

Using the newly developed miRNA screening system, we set out to screen for miRNAs that target the 3'UTR of Oct4 gene, a stem cell transcription factor. Embryonic stem cells (ESCs) are valuable resources for clinical therapies because of their unlimited self-renewal ability and potential to generate any differentiated cell type (pluripotency). The self-renewal and pluripotency properties are regulated by an array of protein-coding genes, such as transcription factors and chromatin remodeling enzymes, in a core regulatory circuitry (Boyer et al., 2005). This circuitry includes Oct4, which forms self-regulatory networks with other stem cell transcription factors and controls a wide range of downstream genes. Extensive studies have indicated that Oct4 (Niwa et al., 2000) is required for ESC self-renewal and pluripotency. Thus, the overexpression of Oct4 can reprogram or dedifferentiate somatic cells into iPSCs in both mice (Nakagawa et al., 2008) and humans (Park et al., 2008).

**1.3. The importance of Oct4 in cancer stem cells (CSC)**

Oct4 (also known as Oct3, Oct3/4, and POU5F1), a member of the family of POU homeodomain domain transcription factors, is expressed in pluripotent embryonic stem and germ cells. Oct4 is primarily expressed in early cleavage stage, inner cell mass, primitive ectoderm, primordial germ cells, and also in embryonic stem, embryonic germ, and embryonic carcinoma cells (Kim et al., 2009). Oct4 is a transcriptional regulator that can activate or repress target gene expression, depending on the cellular context. Oct4 activates transcription via octamer motifs, and Oct4 binding sites have been found in various genes, including fibroblast growth factor 4 (*fgf 4*) and platelet-derived growth factor α receptor (*pdgfαr)* (Lamb & Rizzino, 1998; Kraft et al., 1996). Additionally, Oct4 mRNA is present in fertilized occytes and early embryos, and its expression is maintained until mid-gastrulation at which point it disappears (Nichols, 1998). This suggests that proper levels of Oct4 expression are critical for maintenance of pluripotency and stem cell phenotype (Niwa et al., 2000). In this way, Oct4 functions as a master switch during differentiation by regulating the pluripotent potentials of ESC (Grinnell et al., 2007). Previous reports have also detected Oct4 expression in germ cell tumors (Gidekel et al., 2003) but, more

surprisingly, its expression has recently been detected in somatic-cell tumors (reviewed by Hochedlinger et al., 2005), suggestting that reactivation of Oct4 expression may be associated with cancer stem cell (CSC) biology.

Within a tumor, only certain cells may have the ability to divide uncontrollably and generate new tumors, and such cells are cancer stem cells (Lodish, 2008). A consensus panel convened by the American Association of Cancer Research has defined a CSC as "a cell within a tumor that possesses the capacity to self-renew and cause the heterogeneous lineages of cancer cells that comprise the tumor" (Clarke et al., 2006). Because stem cells can divide continually over the life of an organism, oncogenic mutations in their DNA can accumulate, eventually transforming them into cancer stem cells. CSCs use the same mechanism as ESCs to proliferate and divide uncontrollably. Stem cells that have acquired these mutations have an abnormal proliferative capability and generally cannot undergo normal processes of differentiation (Croker et al., 2008). Many oncogenic mutations, such as those that prevent apoptosis or generate an inappropriate growth-promoting signal, can also occur in more differentiated, but still replicating, progenitor cells (Lodish, 2008). Based on reports that Oct4 increases the malignant potential of ESC in a dose-dependent manner, a possible oncogenic role was attributed to Oct4 (Gidekel et al., 2003). In addition to protein-coding genes, miRNAs are connected to the transcriptional regulatory of ESCs (Marson et al., 2008). Therefore, one possible form of gene therapy against tumors involves upregulating miRNA expression to the Oct4 site to effectively block expression of this CSC gene, which will cease cancer cell proliferation at the tumor site. This line of reasoning provides the basis for the desire to screen for miRNAs that target Oct4-3'UTR in this study.

### 1.4. The importance of screening miRNAs that target Oct4

It is evident that miRNAs play an important role in affecting stem cells and CSC. Some miRNAs, such as miR-290 and miR-302, are found to be inducers of the iPSC generation (Lin et al., 2010). Another similar study revealed a new miRNA family (miR-130/301/721) as an important regular of iPSC induction by

targeting the homeobox transcription factor Meox2 (also known as Gax), the knockdown of which facilitates the overexpression of Oct4, Sox2, and Klf4 stem cell transcription factors, all of which inhibit cell differentiation (Pfaff et al*.,* 2011). Murine embryonic stem cells (ESCs) with genetic deletion of key miRNA processing enzymes Dicer (Kanellopoulou et al., 2005) or DGCR8 (Wang et al., 2007) lose their pluripotency and show defective differentiation. Dicer-deficient mutant ESCs can be partially rescued by the mir-290 cluster miRNAs that regulate Rbl2-dependent DNA methylation to downregulate Oct4 indirectly (Benetti et al., 2008; Sinkkonen et al., 2008). However, mir-290 is found to decrease during ESC differentiation, when Oct4 needs to be switched off, which makes it less likely to become a dominant player in the differentiation process. Nevertheless, the study of the functions of miRNAs in ESCs is still in its early stage, and the direct roles of known human miRNAs in downregulating pluripotency genes are yet to be investigated.

In this study, based on the discussed importance of Oct4 in CSC development, Oct4 was chosen as the initial target gene of interest for the miRNA screening system. Oct4's small gene size of 1082 base pairs (bp) with a relatively short 3'UTR (264 bp) makes it a good candidate for the initial screening of the newly developed system; its small gene size would also allow for easy set-up and modification of the screening parameters and conditions. Using the novel miRNA screening system, we set out to investigate the roles of miRNA in the direct repression of the 3'UTR of Oct4. In a similar objective, Xu et al., using luciferase assay to screen a total of 466 human miRNAs, identified Oct4, along with two other pluripotency factors, SOX2 and KLF4, as a direct target of miR-145 and show that endogenous miR-145 represses the 3'UTR of Oct4, Sox2, and Klf4 in both Hela cells and human ESCs (2009).

In this study, the aim is to screen a bigger human miRNA library of 931 plasmids purchased from GeneCopeia Inc and approach the profiling of the miRNAs against Oct4 3'UTR through a novel method that combines robust small molecule screening technology and standard transfection protocols. It was hypothesized that, besides the already known miR-145, at least one new miRNA from a library of 931 miRNAs will be identified to downregulate Oct4 gene expression by directly targeting its 3'UTR. The

hypothesis is sensible because, with a library of 931 miRNAs, there are likely to be undiscovered miRNAs that downregulates Oct4 gene expression. Also, this study uses a novel screening approach that has the potential to be better at narrowing down candidate miRNA targets than previous screening methods. Consequently, if the 3'UTR of Oct4 is targeted for downregulation by a miRNA, then, based on the imaged data of the DsRed2 fluorescence intensities, its DsRed2 protein expression would be significantly decreased in comparison to highest level of DsRed2 expression intensities and similar to the lowest level of DsRed2 expression intensities. Quantitatively, if a miRNA is a candidate against Oct4-3'UTR, then it should have equal to or more than 50% DsRed2 protein inhibition. Since Oct4 regulation in CSC is similar to that in normal stem cells, it is believed that the characterization of the miRNA pathways and their underlying molecular mechanisms in regulating stem cell genes, such as Oct4, is of great importance to the understanding of ESC self-renewal and pluripotency, which will lead to a better understanding of CSC characterization.

## 2. Materials and Methods

### 2.1.Cell Culture

Human HEK 293FT cells were maintained in Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 10% fetal bovine serum (FBS) and antibiotics at 37°C with 5% carbon dioxide.

### 2.2. miRNA expression plasmids

The 931 miRNA expression plasmids, two of which are mock controls containing no miRNA, were purchased in *E. coli* from GeneCopoeia.

### 2.3. pDsRed2 reporter construct with wild-type Oct4 3' UTR

The Oct3/4-3'UTR was amplified by PCR from human genome DNA using primers Oct4 3'UTR forward GGAAGATCTTGAGGTGCCTGCCCTTCT and reverse CCGCTCGAGTAAGTGTGTCTATCTACTGTGTCCCAGGC. The Oct4 3'UTR DNA segment was then digested using BgLII and Xho I, and ligated into pDsRed2-C1 vector (Clontect) to generate pDsRed2-Oct3/4-3'UTR. The sequences of all of the new constructs were confirmed by sequencing.

### 2.4. pCMV-HA reporter construct with Oct4 3'UTR

The Oct3/4-3'UTR was amplified by PCR from human genome DNA using primers Oct4 3'UTR forward GGAAGATCTTGAGGTGCCTGCCCTTCT and reverse CCGCTCGAGTAAGTGTGTCTATCTACTGTGTCCCAGGC. The Oct4 3'UTR DNA segment was then digested using EcoR I and Not I, and ligated into pDsRed2-C1 vector (Clontect) to generate pDsRed2-Oct3/4-3'UTR. The sequences of all of the new constructs were confirmed by sequencing.

**2.5. Plasmid Purification**

The plasmids were purified using the QIAGEN Plasmid Mini Kit. Each plasmid was diluted in Opti Medium to the desired concentration.

**2.6. Transfection**

Human 293FT cells were plated by the Multi-drop combi bulk dispenser robot (ECBDC) at the desired cell density in 100ul DMEM medium/well in 96-well plates. 24 hours after seeding, cells were transfected with miRNA expression plasmid and of pDsRed2 Oct4 3' UTR reporter plasmid with 0.5ul Lipofectamine Transfection Reagent by the Caliper Sciclone ALH3000 Workstation liquid handler. Images and the total integrated intensities of the pDsRed2-Oct4-3'UTR protein and FITC miRNA expressions were read after 72 hours of transfection by the Molecular Devices ImageExpress Automated cell imaging and analysis system.

**2.7. Western Blotting**

Standard procedures were followed for Western blots using whole cell extracts. Whole cell lysates from cells were obtained by lysing cells with radioimmunoprecipitation assay buffer (RIPA) supplemented with protease inhibitor mixture for 30 min at 4°C. The proteins were separated by polyacrylamide gel electrophoresis (SDS-PAGE). Proteins were transferred onto a polyvinylidene difluoride (PVDF) membrane (BioRad). Membranes were blocked with 10 mM Tris (pH 7.5), 150 mM sodium chloride, and 0.1% Tween 20 with 5% non-fat milk and 0.5% BSA and blotted with the antibodies B-actin (sc-4778, Santa Cruz Biotechnology) and HA-probe (sc-805, Santa Cruz Biotechnology). Blots were then stained with secondary antibodies IRDye Goat anti-Rabbit (926-32221, Li-Cor) and Goat anti-Mouse (926-32210, Li-Cor) and visualized using Li-Cor Odyssey. β-actin was used as the loading control.

**2.8. Data and Statistical Analysis**

The Molecular Devices ImageExpress Automated cell imaging and analysis system was used to image the transfected wells at all three wavelengths for DAPI, GFP, and DsRed2. Metamorph analysis softwared was used to quantify the total fluorescence of each well read for both the DsRed2 and GFP channels. The DAPI staining and resulting signal was used by the analysis software to identify cells so that quantified DsRed2 and GFP signals were only included if they were associated with a viable (nuclear intact) cell. Hence, the output of DsRed2 and GFP intensities were total fluorescence generated by transfected, viable cells only.

The quantified results of the total fluorescence of each well included % Control and % Inhibition values for both the DsRed2 and GFP channels. Each of the % Control values were calculated by first averaging the integrated fluorescence value for all of the miRNA wells on the plate and then dividing the specific well value by this control average and multiplying by 100. Specifically:

%Control(DsRed) = ((miRNA DsRed value)/(average DsRed value for all miRNA wells on plate))*100.

This calculation is the same for the FITC values. The % inhibition values are calculated by simply subtracting the % control value from 100. For example, if the calculated % Control for a given well is 80%, then the corresponding % inhibition value is 20%. All of these calculations were done on a plate-by-plate basis to compensate for any differences in transfection or expression levels from plate to plate. The Z Factor is a common parameter that is calculated for screening plates as a measure of how consistent and robust an assay is. It takes into account the signal window (difference between the negative and positive control wells) as well as the standard deviations of each of these values. Generally, a Z Factor of > 0.5 is considered acceptable for screening, though that criterion is relaxed in the case of imaging data. The Z-factor is defined in terms of four parameters: the means and standard deviations of both the positive (p) and negative (n) controls ($\mu_p$, $\sigma_p$, and $\mu_n$, $\sigma_n$). Given these values, the Z-factor is defined as:

$$\text{Z-factor} = 1 - \frac{3(\sigma_p + \sigma_n)}{|\mu_p - \mu_n|}.$$

## 3. Results

### 3.1 Development of the miRNA screening system

*3.1.1. miRNA expression plasmid purification and dilution*

Of the 931 miRNA expression plasmids, two are mock controls, encoding no miRNA. Each of the miRNA coding sequence is attached to the 3' end of eGFP coding sequence, which has a strong CMV promoter (**Fig. 1a**). The E. coli with the miRNA expression plasmids were cultured overnight in Luria Broth (LB) medium with ampicilin and the plasmids were purified from the E.coli bacteria culture (**Fig. 1b**). The concentrations of each of the 931 plasmids were measured in using Nanodrop 1000 (ThermoScientific). Each plasmid was diluted in Opti Medium (Gibico) to the desired concentration specific for each transfection. Each diluted plasmid was aliquot to 250ul in each well in round-bottom 96 well plates for mass automatic transfection. The edge wells were not used because previous experimental data showed that there were severe edge effects in DsRed2 protein expression, perhaps resulting from media evaporation and/or uneven cell seeding, of transfections in these outer wells (data not shown). Each plate contains 56 miRNA samples and 4 mocks, resulting in 17 diluted 96-well plates for all 931 miRNAs (**Fig. 1d**). The plasmid dilution map for plate 1 shows the basic dilution map of the plasmids representative of each of the 17 plates (**Fig. 1c**). To confirm the miRNA expressions of these purified plasmids, some plasmids were randomly selected and transfected into 293FT cells. The GFP signals for these plasmids were clearly detected, which means that these plasmids' qualities are sufficient for mammalian cell transfection and the miRNAs' expression levels are rather high (**Fig. 1e**).

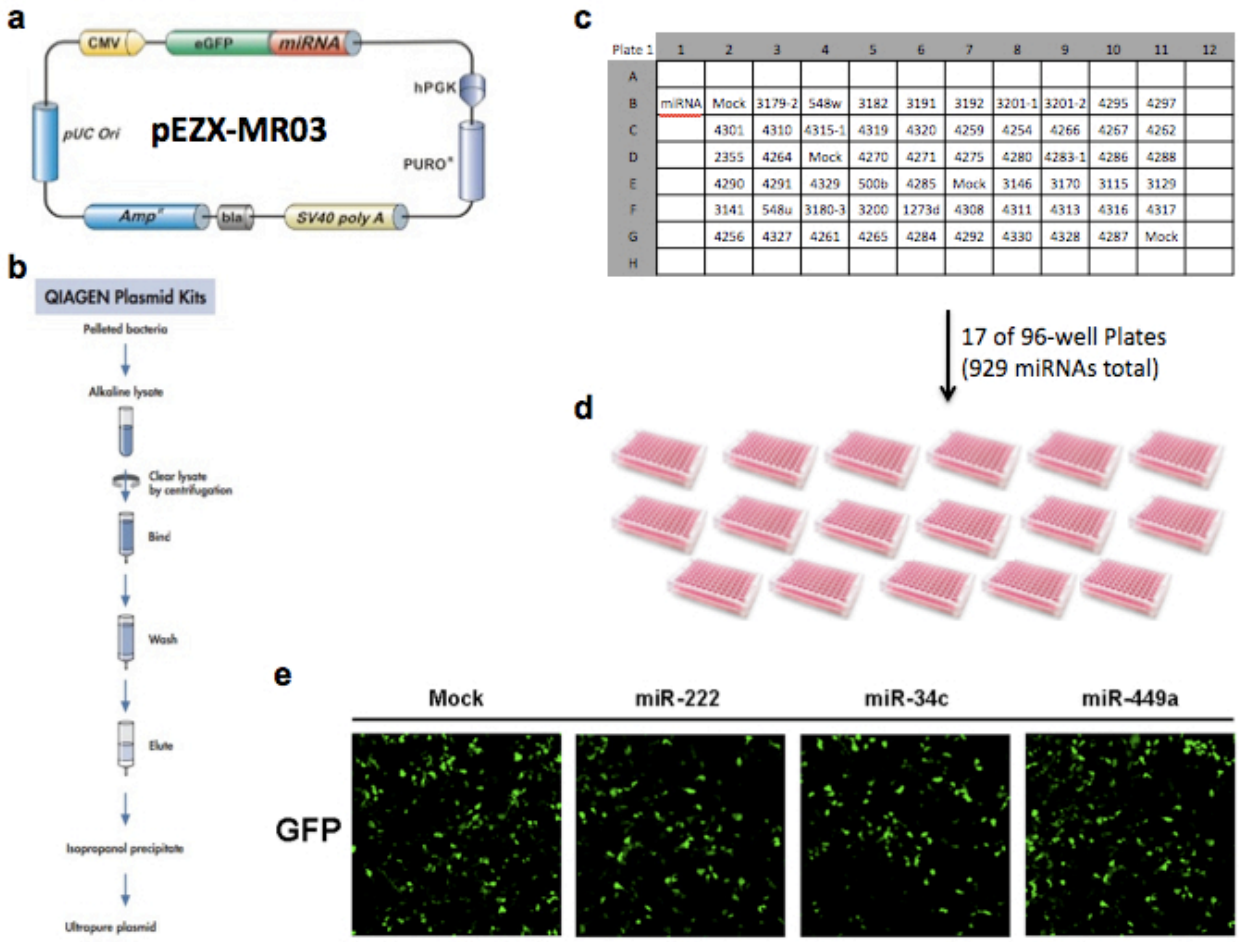



**Figure 1. miRNA expression plasmid**. (**a**) miRNA plasmid construct of miRNA transcript attached to eGFP coding sequence. (**b**) miRNA plasmid purification protocol (**c**) Plate 1 dilution map of miRNA plasmids in a round-bottomed 96-well plate, containing 56 miRNA samples and 4 mocks. The outer wells of the plate were not used to avoid edge effects. (**d**) 931 miRNA plasmids diluted in 17 96-well plates. (**e**) GFP expressions of mock and three randomly selected miRNAs after 48hrs after transfections into 293FT cells.

### *3.1.2. Optimize HEK 293 FT Seeding Density*

To determine the suitable initial cell seeding concentration that reaches around 70% confluency after 24 hours of seeding for transfection, cells were seeded at 40,000, 20,000, 10,000, and 5,000 cells per 100ul in 96 well plates. The cell images after 24 hours show that the initial cell seeding at 20,000 cells per well can reach nearly 70% confluency after 24 hours (**Fig. 2**). Cells were seeded at this concentration for the following transfection experiments.

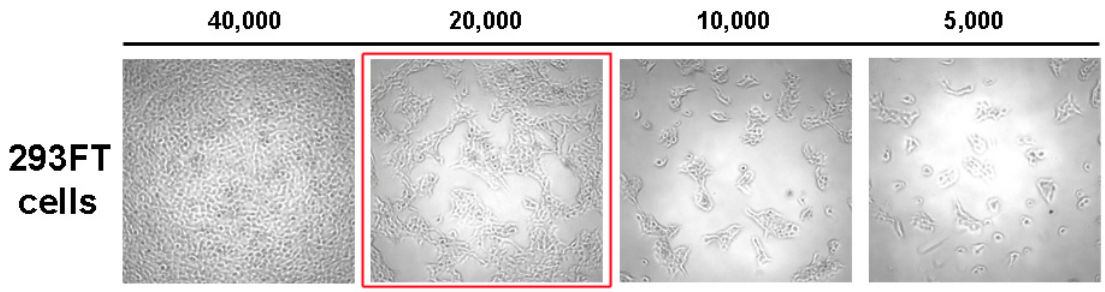



**Figure 2. 293FT cells confluencies after 24 hours of initial seedings at different densities.** For 40,000 cells/well, cells reached nearly 100% confluency after 24 hours, ~70% for 20,000 cells/well, ~40% for 10,000 cells/well, and ~30% for 5,000 cells/well.

*3.1.3. Construction of pDsRed2-Oct4-3'UTR plasmid*

The pDsRed2-Oct4-3'UTR was generated by inserting the Oct4-3'UTR, which is 264 base pairs (bp) in length, into the expression vector pDsRed2-C1 (Clontech) (**Fig. 3a**). The new construction was first confirmed by gel electrophoresis. The Oct4-3'UTR band, as predicted, fell between 0.3 and 0.2 kilo base pairs (kb) or 300 and 200 bp (**Fig. 3b**). Then it was confirmed by sequencing. Next, to examine the DsRed2 protein expression, pDsRed2-Oct4-3'UTR plasmid was transfected into 293FT cells; after 48 hours of transfection, DsREd2 protein expression was clearly detected **(Fig. 3c**).

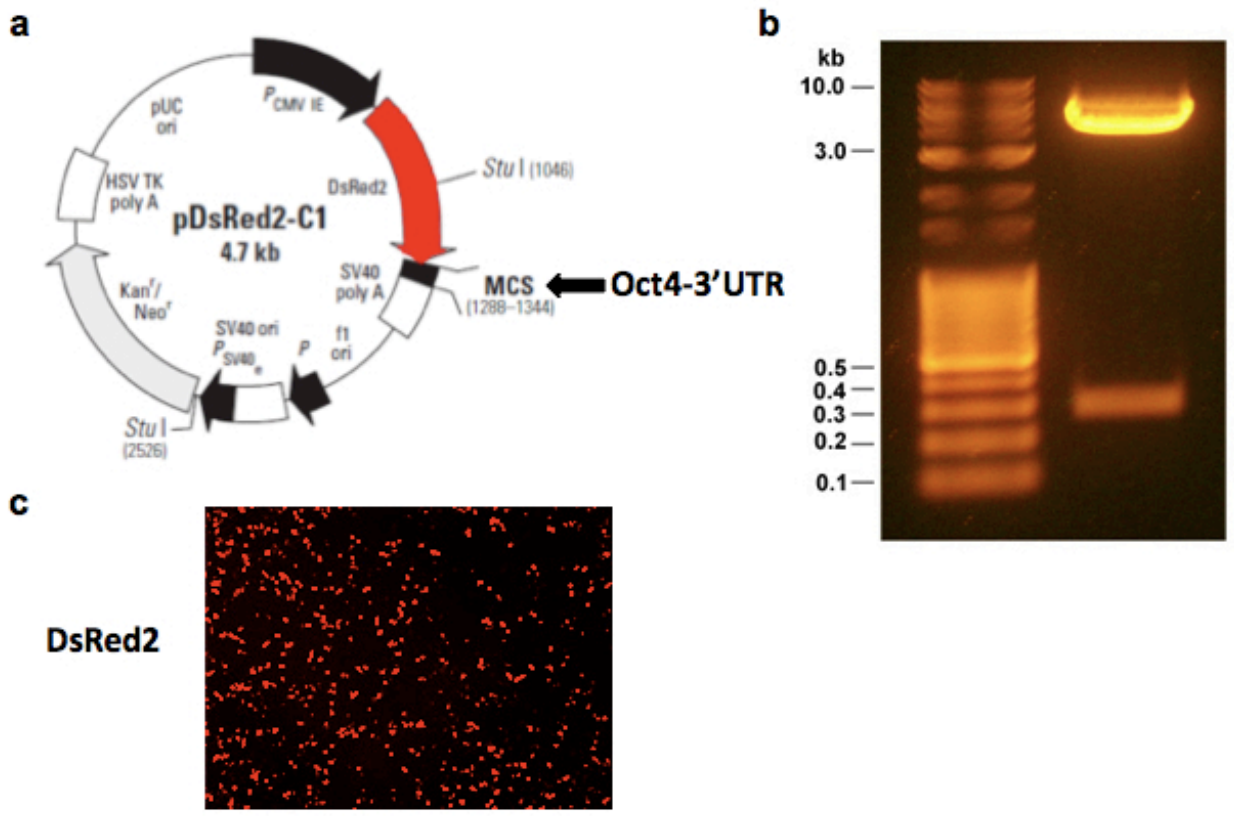



**Figure 3. pDsRed2-Oct4-3'UTR plasmid.** (**a**) The Oct4-3'UTR was inserted at the multiple clone site (MCS) of the pDsRed2-C1 plasmid. (**b**) Gel electrophoresis confirmed the Oct4-3'UTR (264 bp) band between 0.3 and 0.2 kp. (**c**) DsRed2 protein expression after 48 hrs of p-DsRed2-Oct4-3'UTR transfection into 293FT cells.

*3.1.4. Determine the optimal parameters for transfecting the pDsRed2-3'UTR plasmid*

To determine the optimal parameters for transfecting the pDsRed2-3'UTR plasmid, varying concentrations of pDsRed2-Oct4-3'UTR and pDsRed2-p53-3'UTR were transfected. pDsRed2-p53-3'UTR was used for the initial set-up of the screening system because the construct is readily available in Wang's lab and p53 has been extensively researched on miRNA regulation; hence, p53 is a suitable gene to use as a positive control for the initial set-up of this novel screening system. Transfections at varying DsRed2-3'UTR concentrations yielded dose response curves that helped identified the optimal transfection concentration for suitable DsRed2 protein expression level without severe cell toxicity and extreme ranges of DsRed2 protein expression levels for fluorescence imaging detection. Determining this

optimal concentration enabled for a more sensitive screening system of key miRNAs that could affect the 3'UTR of the target gene.

In the first titration curve, ranges of concentrations from 0 ng to 200 ng of pDsRed2-p53-3'UTR and pDsRed2-Oct4-3'UTR were transfected into 293 FT cells. ImageExpress (ECBDC) captured the DsRed protein expressions of these transfections. The results showed that as the concentration of the pDsRed2-3'UTR plasmids increased, the DsRed expression signal became stronger and reached a plateau when the concentration is about 50 ng for both titrations (**Fig. 4a**). Metamorph analysis software (ECBDC) quantified the DsRed2 expression intensities in pixel counts; the intensities exponentially increased from 0 to 50 ng and leveled off at 20,000 and 35,000 pixels for pDsRed2-p53-3'UTR and pDsRed2-Oct4-3'UTR, respectively (**Fig. 4d,e**). As also seen in the DAPI (4',6-diamidino-2-phenylindole) staining, there are the most live cells in untransfected (0 ng) regions, less live cells in concentrations between 3 ng and 50 ng, and the least amount of live cells from 50 ng and higher concentrations (**Fig. 4b**). These results suggested that concentrations higher than 50 ng pDsRed2-3'UTR can induce severe cell toxicity. Hence, in the 2$^{nd}$ titration curve, the concentration range was narrowed down to varying intervals between 0 ng and 40 ng for DsRed2-3'UTR of Oct4 and p53. The DsRed2 protein expression signals increased exponentially as the concentration increases (**Fig. 4c)**. For the purpose of this screening system, a concentration that expresses 50-60% DsRed expression level after 72 hrs after transfection is considered the optimal concentration for transfection protocols. This concentraion would allow for above and below ranges of DsRed2 protein expression levels to be detected by the fluorescent microscope and image capture without inducing cell toxicity. The dose response curves identified 8 ng of pDsRed2-3'UTR plasmid (for each well of 96-well plate) as the optimal concentration for all the following transfections (**Fig. 4f,g**).

In both titration curves of pDsRed2-p53-3'UTR and pDsRed2-Oct4-3'UTR plasmids, the latter DsRed2 protein expression intensity was consistently stronger than the former (**Fig. 4a,c top vs. bottom panels**). The difference in their transfection efficiencies might be due to their lengths; the 3'UTR of p53

is 1191 base pairs (bp) in length, while the 3'UTR of Oct4 is 264 bp. The shorter pDsRed2-Oct4-3'UTR sequence has a higher efficiency of inserting into the nucleus and incorporating itself into the 293FT cells' DNA, and therefore, increases its DsRed2 protein expression, than the longer pDsRed-p53-3'UTR. Their degree of regulations by other proteins, transcription factors, and endogenous miRNAs could also explain the difference in their intensities. Since p53 has a longer 3'UTR than Oct4, it is also more prone to regulations by these factors in vivo than Oct4, decreasing its DsRed2 protein expression, and thus, its observed intensity.

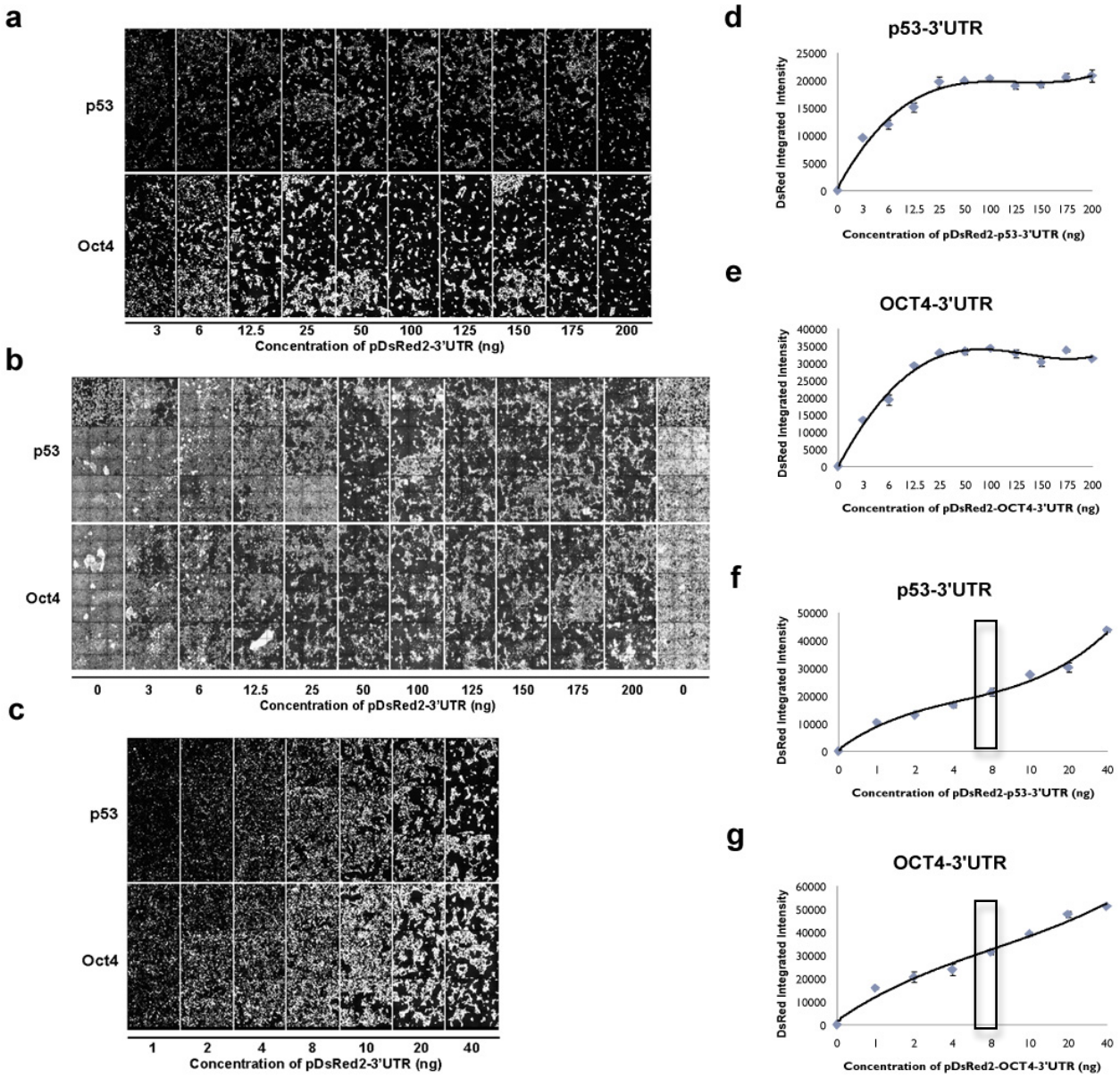



**Figure 4. Dose response transfections of pDsRed2-3'UTR of p53 and Oct4.** (**a**) DsRed2 protein expression image of 1st dose response titration after 72 hours of transfections of pDsRed2-p53-3'UTR (top panel) and pDsRed2-Oct4-3'UTR (bottom panel) at varying concentrations between 3 ng and 200 ng. (**b**) DAPI staining image of the 1st titration shows severe cell toxicity from 50 ng and above. (**c**) DsRed2 protein expression image of 2nd titration curve after 72 hours of transfections at varying concentrations between 1 ng and 40 ng. (**d,e**) 1st Dose response curves of DsRed integrated intensities of p53-3'UTR and Oct4-3'UTR. (**f**,**g**) 2nd Dose response curves of DsRed integrated intensities of p53-3'UTR and Oct4-3'UTR. 8ng (boxed) is determined as the optimal concentration.

*3.1.5. Verification of the reliability of the miRNA screening system*

To verify the reliability of the automatic screening system, p53 was used as the test target gene since some miRNAs, one of which is miR-1285 (Tian et al., 2010), are known to efficiently target p53 by directly targeting its 3'UTR. 8ng of pDsRed2-p53-3'UTR and 10 ng of several miRNAs, including the mock control, miR-1285, and a randomly selected miRNA, were cotransfected into 293FT cells (**Fig. 5a**). The results show that the DsRed2 protein expression signals were strong in the mock control and the same signal was observed in the randomly selected miRNA with both at intensities around 20,000 pixels (**Fig. 5a,b**). However, the DsRed intensity was dramatically decreased in comparision to the mock and random when co-transfected with mir-1285, which has an intensity of 10,000 pixels (**Fig. 5b**). These results provide strong evidence supporting the reliability of the novel screening system.

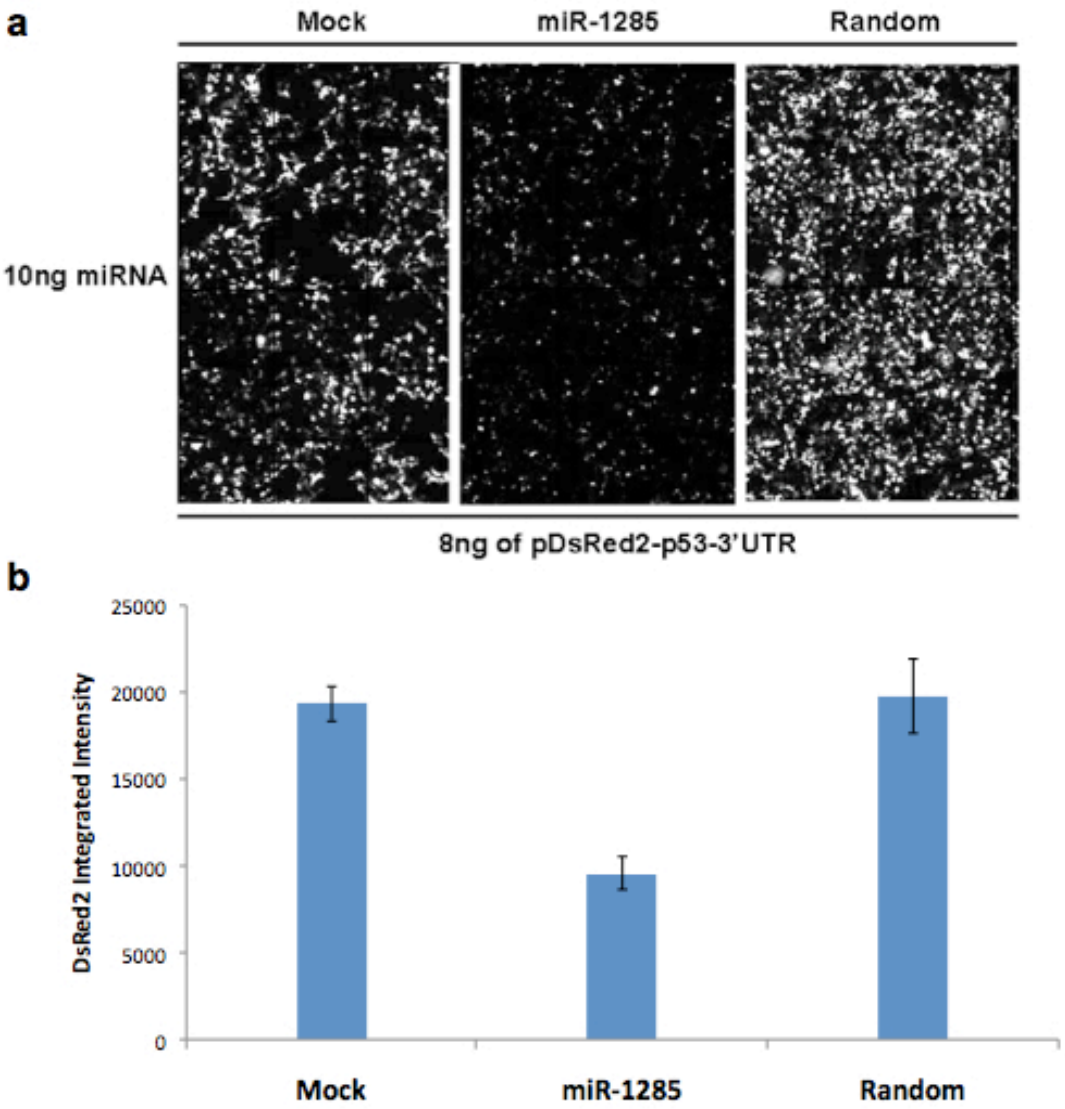



**Figure 5. p53-3'UTR positive control confirmation using the novel miRNA screening system.** (**a**) DsRed protein expression images of co-transfections of 8 ng pDsRed2-p53-3'UTR with 10 ng of mock, miR-1285, and a random miRNA. (**b**) DsRed2 protein expression intensities in pixels count for the three co-transfections.

### 3.2. Screening miRNA that targets Oct4

*3.2.1. Mass miRNA screening of 931 plasmids*

Once the system's screening parameters were identified and the system's reliability was confirmed, a mass co-transfection of pDsRed2-Oct4-3'UTR with each of the 931 plasmids were performed in 293FT cultured 96-well plates. The first and last column wells of each plate were transfected with only 0.5 ng

and 8.0 ng pDsRed2-Oct4-3'UTR, respectively, for cut off value calculations to define the range of DsRed2 expression (**Fig. 6a)**. The 0.5 ng transfections of these wells are nearly the lowest DsRed2 expression intensity and, ideally, the miRNAs that can effectively downregulate pDsRed2-Oct4-3'UTR expression will have similar range of low DsRed2 expression intensities. Through the same line of reasoning, the DsRed2 protein expression intensities in the 8.0 ng transfections are the highest; the miRNAs that have no inhibitory regulation on the Oct4-3'UTR will have similar expression intensities.

The mass miRNA screening was divided into two sets, one of 479 miRNAs and the other of 450 miRNAs. From the two mass transfections, images of DAPI, GFP, and DsRed2 signals were captured for all the miRNA and DsRed2-Oct4-3'UTR co-transfected wells. The transfection map and DsRed and GFP protein expression fluorescence images of the plate 1 are shown in **Figure 6** as a representative example of the 17 transfected plates. The DAPI staining images of the 17 transfected plates of the 931 miRNAs show that 293 FT cells grew evenly without any cell toxicity or contamination (images not shown). The GFP fluorescent image represents miRNA expression and, thus, the signal of each well reflects the transfection efficiency of the corresponding miRNA (**Fig. 6b**). The DsRed2 flourescence images were used to visually indicate the miRNAs that could downregulate Oct4-3'UTR (**Fig. 6c**). The miRNAs of the corresponding wells with similar high level of DsRed2 intensities as the 8.0 ng DsRed-Oct4-3'UTR transfections, as in miR-4265 in G5, were eliminated as potential negative regulators of Oct4-3'UTR. In contrast, miRNAs of corresponding wells with low DsRed2 intensities, similar to the 0.5 ng DsRed-Oct4-3'UTR transfected wells, as in miR-4310 in C3, were identified as potential negative regulators of Oct4-3'UTR (**Fig. 6a,c).**

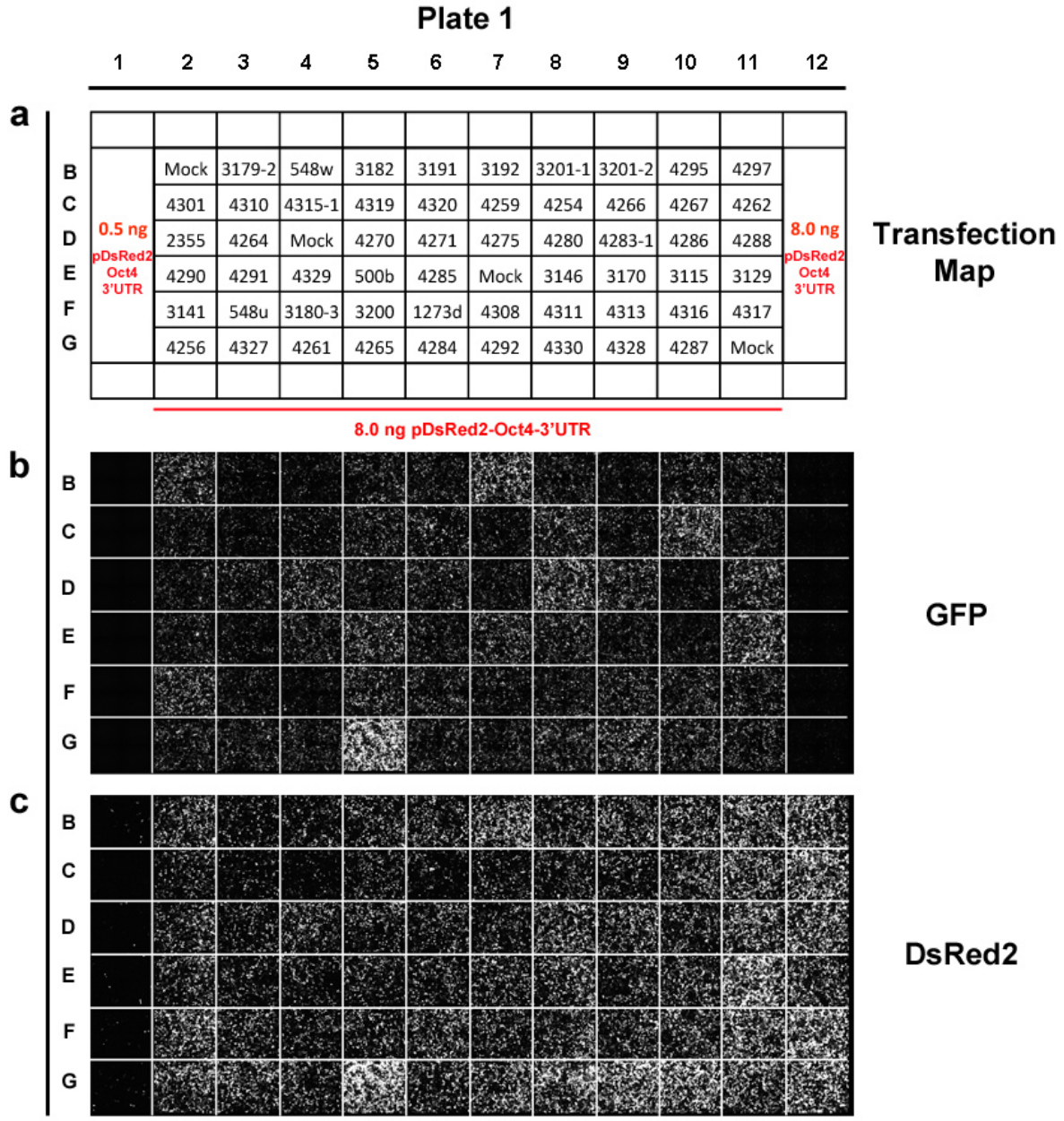



**Figure 6. Plate 1 of miRNA and pDsRed2-Oct4-3'UTR co-transfections.** (**a**) Transfection map for plate 1 of 17. Columns 2-11 and Rows B-G were co-transfected with 8 ng pDsRed2-Oct4-3'UTR and 10 ng of the corresponding miRNA expression plasmid. Columns 1 and 12 (Rows B-G) were transfected with only pDsRed2-Oct3-3'UTR at 0.5 ng (positive controls) and 8 ng (negative controls), respectively. (**b**) Image of GFP protein fluorescence intensity of transfected wells in plate 1. Columns 1 and 12 did not express any GFP signals because these wells were not transfected with GFP proteins containing miRNA transcripts. (**c**) Image of DsRed2 protein florescence intensity of transfected wells in plate 1. Column 1 shows very low DsRed2 intensity from only 0.5 ng pDsRed2-Oct4-3'UTR transfection, while column 12's high DsRed2 intensity reflects transfection at 8.0 ng.

In additional to the raw imaged data, statistical analysis of DsRed2 and GFP/miRNA protein expression intensities was also analyzed for the 17 co-transfected plates. The Metamorph software analysis (ECBDC) counts the total pixels of the grayscale images of GFP and DsRed2; the total pixel counts reflect the total integrated intensity of the GFP and DsRed2 expressions. The miRNAs of the two sets of mass transfections were separately ranked according to DsRed2 % inhibitions (**Fig. 7a,c**). The miRNAs above the x-axis have some kind of an inhibitory effect of Oct4-3'UTR, while the miRNAs below the x-axis have no or up-regulatory effects on Oct4-3'UTR (**Fig. 7a,c**). This study's cut-off DsRed2 % inhibition was set at 50% to narrow down the potential miRNA candidates that have inhibitory effects on Oct4-3'UTR expression. Of the 479 miRNAs screened in the first mass transfection, 57 miRNAs made the cut off (**Fig. 7b**), and 27 miRNAs made the cut off from the second mass transfection of 450 miRNAs (**Fig. 7d**).

Consequently, based on DsRed % inhibition data analysis, 84 out of a library of 931 human miRNAs were identified as potential negative regulators of Oct4 gene by directly binding to its 3'UTR (**Table 1**). The miRNA with the most inhibitory effect on Oct4-3'UTR with 89.72% DsRed2 inhibition was miR-4315 from the 1$^{st}$ mass transfection. DsRed2 % inhibition calculations and rankings correlate with the raw imaged data of DsRed2 protein expression intensities, as in miR-4315 (**Fig. 7e**). The correlation between the raw imaged data and the quantified data analysis suggests that the data are reliable for the purpose of narrowing down a library 931 human miRNAs for potential candidates that can regulate Oct4-3'UTR. It should be noted that this system did not confirm Xu et al.'s results that miR-145 repressed the 3'UTR of Oct4 (2009). Instead, this system's screening found that miR-145 has no inhibitory effect on Oct4-3'UTR (**Fig. 7e**).

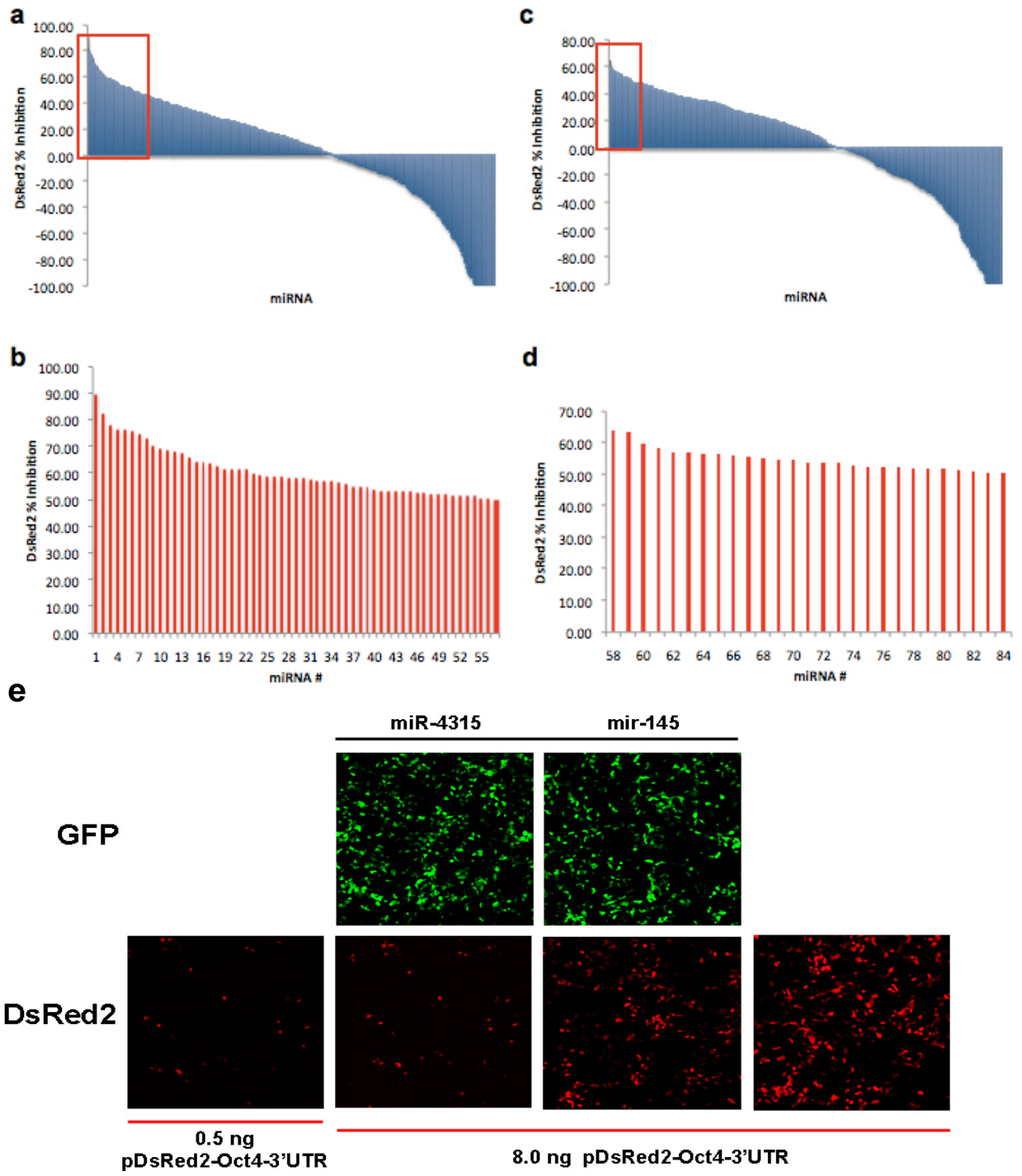



**Figure 7. 931 miRNAs pDsRed2 % inhibition rankings and raw imaged data.** (**a**) DsRed2 % inhibition rankings of 479 miRNAs in the 1st mass co-transfection. (**b**) DsRed2 % inhibition rankings of 57 miRNAs above the cut off values of 50% inhibition in the 1st mass transfection. (**c**) DsRed2 % inhibition rankings of 450 miRNAs in the 2nd mass transfection. (**d**) DsRed2 % inhibition rankings of 27 total miRNAs above the cut off values of 50% inhibition for the 2nd mass transfection. (**e**) Downregulation of DsRed2-Oct4-3'UTR expression was observed for miR-4315, but not for miR-145, when compared to the highest and lowest DsRed2 expression intensities.

**Table 1. miRNA with ≥50% DsRed2 Inhibition**

| miRNA # | miRNA ID | DsRed | GFP | DsRed (%Ctrl) | FITC (%Ctrl) | DsRed % Inh |
|---|---|---|---|---|---|---|
| *1 | 4315 | 1.08E+08 | 2.12E+08 | 10.28 | 34.40 | 89.72 |
| 2 | 548i-3 | 2.07E+08 | 2.31E+08 | 17.52 | 40.26 | 82.48 |
| 3 | 548i-1 | 2.57E+08 | 2.06E+08 | 21.71 | 35.90 | 78.29 |
| 4 | 1321 | 2.78E+08 | 3.19E+08 | 23.50 | 55.64 | 76.50 |
| 5 | 4310 | 2.50E+08 | 2.06E+08 | 23.74 | 33.34 | 76.26 |
| 6 | 548h-4 | 2.85E+08 | 2.68E+08 | 24.10 | 46.83 | 75.90 |
| 7 | 4320 | 2.67E+08 | 4.68E+08 | 25.36 | 75.76 | 74.64 |
| 8 | 585 | 4.95E+08 | 1.49E+08 | 27.08 | 37.90 | 72.92 |
| 9 | 362 | 5.44E+08 | 1.76E+08 | 29.76 | 44.67 | 70.24 |
| 10 | 299 | 3.63E+08 | 4.43E+08 | 30.67 | 77.30 | 69.33 |
| 11 | 3156-1 | 3.72E+08 | 3.15E+08 | 31.41 | 55.05 | 68.59 |
| 12 | let-7f | 5.74E+08 | 2.02E+08 | 32.14 | 51.19 | 67.86 |
| 13 | 3166 | 3.83E+08 | 2.28E+08 | 32.35 | 39.85 | 67.65 |
| 14 | 608 | 7.81E+08 | 3.44E+08 | 34.21 | 54.48 | 65.79 |
| 15 | 26a-1 | 8.09E+08 | 1.88E+08 | 35.46 | 29.80 | 64.54 |
| 16 | 4273 | 4.26E+08 | 2.83E+08 | 35.96 | 49.32 | 64.04 |
| 17 | 877 | 6.38E+08 | 2.54E+08 | 36.25 | 57.37 | 63.75 |
| 18 | 1259 | 6.53E+08 | 2.65E+08 | 37.12 | 59.92 | 62.88 |
| 19 | 3160 | 6.12E+08 | 3.64E+08 | 38.21 | 52.76 | 61.79 |
| 20 | 3179-2 | 4.06E+08 | 1.97E+08 | 38.53 | 31.89 | 61.47 |
| 21 | 181a-2 | 8.82E+08 | 1.52E+08 | 38.63 | 24.02 | 61.37 |
| 22 | 1184 | 7.07E+08 | 3.15E+08 | 38.66 | 80.03 | 61.34 |
| 23 | 2053 | 9.12E+08 | 2.27E+08 | 39.98 | 35.91 | 60.02 |
| 24 | 192 | 9.25E+08 | 4.10E+08 | 40.52 | 64.93 | 59.48 |
| 25 | 889 | 7.54E+08 | 2.89E+08 | 41.23 | 73.32 | 58.77 |
| 26 | 3154 | 4.89E+08 | 5.07E+08 | 41.36 | 88.48 | 58.64 |
| 27 | 1207 | 7.41E+08 | 2.68E+08 | 41.47 | 68.11 | 58.53 |
| 28 | 3156-2 | 5.35E+08 | 3.49E+08 | 41.50 | 72.48 | 58.50 |
| 29 | 891-b | 7.63E+08 | 2.75E+08 | 41.74 | 69.66 | 58.26 |
| 30 | 1253 | 9.54E+08 | 2.03E+08 | 41.79 | 32.15 | 58.21 |
| 31 | 3131 | 6.77E+08 | 5.25E+08 | 42.28 | 76.17 | 57.72 |
| 32 | 3128 | 5.05E+08 | 2.58E+08 | 42.70 | 45.09 | 57.30 |
| 33 | 3118-5 | 5.06E+08 | 4.67E+08 | 42.74 | 81.57 | 57.26 |
| 34 | 4329 | 4.52E+08 | 4.10E+08 | 42.88 | 66.45 | 57.12 |
| 35 | 3118-4 | 5.16E+08 | 3.27E+08 | 43.64 | 57.10 | 56.36 |
| 36 | 378c | 5.65E+08 | 3.69E+08 | 43.81 | 76.47 | 56.19 |
| 37 | 4291 | 4.72E+08 | 2.62E+08 | 44.82 | 42.48 | 55.18 |
| 38 | 4259 | 4.73E+08 | 3.02E+08 | 44.95 | 48.88 | 55.05 |
| 39 | 4319 | 4.77E+08 | 4.82E+08 | 45.30 | 78.12 | 54.70 |
| 40 | 2861 | 7.41E+08 | 6.52E+08 | 46.27 | 94.59 | 53.73 |
| 41 | 3126 | 5.50E+08 | 4.80E+07 | 46.49 | 8.38 | 53.51 |
| 42 | 4264 | 4.90E+08 | 4.01E+08 | 46.53 | 64.97 | 53.47 |
| 43 | 548s | 5.51E+08 | 2.57E+08 | 46.55 | 44.90 | 53.45 |
| 44 | 4275 | 4.91E+08 | 3.20E+08 | 46.64 | 51.84 | 53.36 |
| 45 | 340 | 1.07E+09 | 5.87E+08 | 46.95 | 92.83 | 53.05 |

| | | | | | | |
|---|---|---|---|---|---|---|
| **46** | **4306** | 6.07E+08 | 4.52E+08 | 47.07 | 93.73 | **52.93** |
| **47** | **4301** | 4.99E+08 | 3.30E+08 | 47.36 | 53.43 | **52.64** |
| **48** | **543** | 8.43E+08 | 3.38E+08 | 47.92 | 76.41 | **52.08** |
| **49** | **1185-1** | 1.10E+09 | 3.36E+08 | 47.99 | 53.20 | **52.01** |
| **50** | **639** | 8.45E+08 | 2.01E+08 | 48.02 | 45.46 | **51.98** |
| **51** | **769** | 8.81E+08 | 3.34E+08 | 48.18 | 84.80 | **51.82** |
| **52** | **766** | 1.10E+09 | 3.63E+08 | 48.27 | 57.39 | **51.73** |
| **53** | **548w** | 5.09E+08 | 2.15E+08 | 48.32 | 34.83 | **51.68** |
| **54** | **3118-2** | 5.73E+08 | 4.32E+08 | 48.44 | 75.33 | **51.56** |
| **55** | **519a-2** | 1.58E+09 | 2.96E+08 | 49.31 | 75.23 | **50.69** |
| **56** | **3180-3** | 5.21E+08 | 1.97E+08 | 49.46 | 31.89 | **50.54** |
| ***57** | **183** | 1.60E+09 | 2.53E+08 | 49.90 | 64.22 | **50.10** |
| ****58** | **1468** | 1.34E+08 | 6.50E+08 | 36.14 | 286.15 | **63.86** |
| **59** | **1538** | 1.35E+08 | 8.22E+08 | 36.52 | 361.89 | **63.48** |
| **60** | **130a** | 1.55E+08 | 5.84E+08 | 40.14 | 52.23 | **59.86** |
| **61** | **554** | 1.60E+08 | 8.84E+08 | 41.39 | 79.10 | **58.61** |
| **62** | **520a** | 2.56E+08 | 6.16E+08 | 42.78 | 74.71 | **57.22** |
| **63** | **325** | 1.86E+08 | 8.34E+08 | 42.79 | 91.71 | **57.21** |
| **64** | **663** | 1.67E+08 | 9.50E+08 | 43.38 | 85.02 | **56.62** |
| **65** | **376a-1** | 1.58E+08 | 1.30E+08 | 43.58 | 80.37 | **56.42** |
| **66** | **643** | 2.62E+08 | 7.25E+08 | 43.74 | 87.96 | **56.26** |
| **67** | **548h-1** | 1.82E+08 | 6.86E+08 | 44.36 | 77.39 | **55.64** |
| **68** | **7-2** | 1.96E+08 | 7.42E+08 | 44.93 | 81.62 | **55.07** |
| **69** | **371** | 1.97E+08 | 9.20E+08 | 45.20 | 101.14 | **54.80** |
| **70** | **496** | 1.65E+08 | 1.43E+08 | 45.50 | 88.50 | **54.50** |
| **71** | **487b** | 1.78E+08 | 9.90E+08 | 46.04 | 88.60 | **53.96** |
| **72** | **518d** | 2.76E+08 | 7.69E+08 | 46.20 | 93.21 | **53.80** |
| **73** | **521-1** | 2.77E+08 | 6.06E+08 | 46.27 | 73.48 | **53.73** |
| **74** | **17** | 1.82E+08 | 7.96E+08 | 47.26 | 71.26 | **52.74** |
| **75** | **633** | 2.85E+08 | 8.56E+08 | 47.64 | 103.87 | **52.36** |
| **76** | **29c** | 1.85E+08 | 7.45E+08 | 47.85 | 66.64 | **52.15** |
| **77** | **1261** | 1.61E+08 | 6.73E+08 | 47.85 | 87.10 | **52.15** |
| **78** | **373** | 1.85E+08 | 1.01E+09 | 47.92 | 90.51 | **52.08** |
| **79** | **199b** | 1.77E+08 | 7.86E+08 | 47.92 | 346.26 | **52.08** |
| **80** | **15a** | 1.85E+08 | 1.21E+09 | 47.95 | 108.29 | **52.05** |
| **81** | **548i-4** | 1.63E+08 | 5.43E+08 | 48.54 | 70.23 | **51.46** |
| **82** | **520h** | 2.93E+08 | 9.22E+08 | 49.04 | 111.77 | **50.96** |
| **83** | **596** | 1.83E+08 | 9.31E+08 | 49.46 | 410.01 | **50.54** |
| ****84** | **376c** | 1.80E+08 | 1.20E+08 | 49.67 | 74.41 | **50.33** |

*Candidate miRNAs # 1-57 were identified from the 1st mass transfection set and ranked in decreasing DsRed2 % inhibition among themselves.

**Candidate miRNAs #58-84 were identified in the 2nd mass transfection set and ranked in decreasing DsRed2 % Inhibition among themselves.

*3.2.2. Construction of pDsRed2 Oct4 3'UTR plasmid and western blotting verifications*

To further confirm that the narrowed down miRNAs have inhibitory effects on Oct4 gene by targeting its 3'UTR, only the top 10 miRNAs and 14 random miRNAs (24 in total) from the narrowed down list of 84 miRNAs were selected for confirmation of their downregulation of pCMV-HA-Oct4-3'UTR protein expression by western blotting (highlighted in gray in **Table 1**). First, the pCMV-HA-Oct4-3'UTR was generated by inserting the Oct4 gene with 3'UTR, which is 1.3 kb in length, into the expression vector pCMV-HA (Clontech) (**Fig. 8a**). The new construction was first confirmed by gel electrophoresis. The Oct4 with 3'UTR band, as predicted, fell just below the 1.5 kb ladder band (**Fig. 8b**). Then it was confirmed by sequencing.

For western blotting verification experiments, 293FT cells were co-transfected with pCMV-HA-Oct4-3'UTR with each of the selected 24 candidate miRNAs. After 48 hours after co-transfection, 293FT whole cell extracts were used for western blotting. miR-4310 and miR-1253 were confirmed to have significant downregulation effects on HA protein expression by targeting Oct4-3'UTR (**Fig. 9c,d**). Some miRNAs, such as miR-548h, seem to show downregulation of HA protein expression in the 1st western blot check, but failed to show the same downregulation pattern in the 2$^{nd}$ check (**Fig. 9c,d**); thus, miRNAs that did not show consistent and repeatable results for their downregulation of HA gene expression were eliminated as potential candidates for their inhibitory role against Oct4-3'UTR target gene.

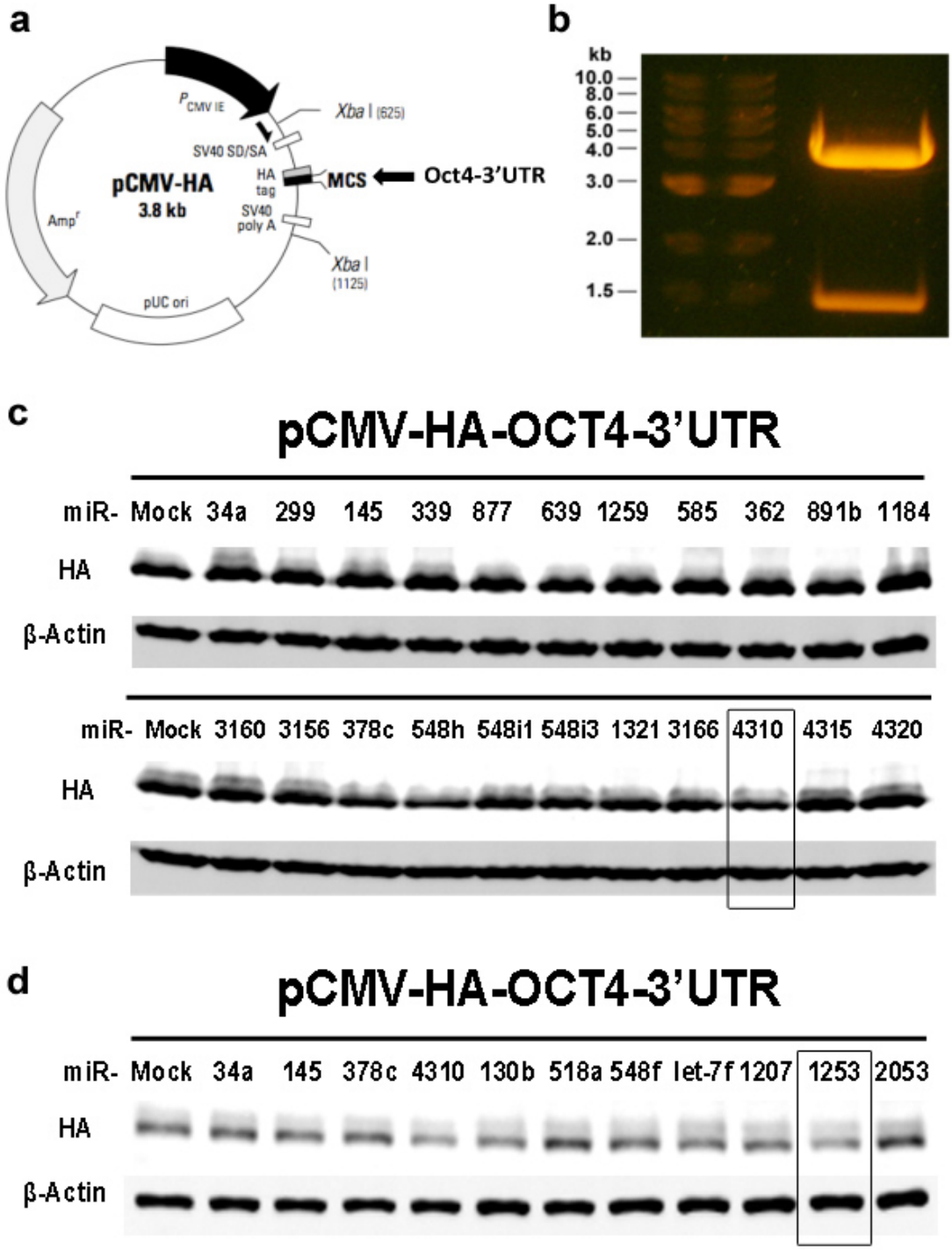



**Figure 8**. **pCMV-HA-Oct4-3'UTR plasmid and western blot verifications.** (**a**) The Oct4-3'UTR was inserted at the multiple clone site (MCS) of the pDsRed2 plasmid. The DsRed protein and Oct4-3'UTR share the same CMV promoter. (**b**) Gel electrophoresis confirmed the Oct4-3'UTR (1.3 kb) band was just below the 1.5 kb ladder band. (**c,d**) Western blotting of HA protein expression verifies miR-4310 and miR-1253 as potential inhibitors of Oct4-3'UTR. β-actin was used as loading controls.

## 4. Discussion

### 4.1. The auto-system provides an efficient approach to screen miRNAs for different purposes

In this study, the objective was to develop a novel, sensitive, and efficient miRNA screening system that combines basic molecular cell biology transfection techniques and multiple integrated robotic systems from the Emory Chemical Biology Discovery Center (ECBDC). Considering that ~1% of the human genome is miRNA genes and most human genes are regulated by at least one miRNA, the potential impact of altered miRNA levels in cellular functions and development is conceivably enormous. Most miRNAs regulate gene expression by binding to the 3'UTR of the target genes; taking advantage of this mechanism of gene regulation, a successful screening system was set up to individually screen 931 miRNA plasmids against any desired 3'UTR of target genes. Optimal parameters, such as cell seeding density, pDsRed2-3'UTR transfection concentration, and transfection time were established to optimize the system. The system's reliability was further confirmed by using p53, a well-known and extensively researched tumor suppressor gene against miRNAs, as a test target during the initial set-up and modifications of the system. As opposed to performing the sensitive screening manually, the utilization of the small molecule modulator discovery and hit optimization robotic systems of ECBDC for the miRNA screening significantly reduced the amount of manual labor, time, and supplies required to individually screen 931 miRNAs.

Using high throughput dispensing methods, such as the MultiDrop combi dispenser to seed cells and the Caliper Sciclone ALH3000 Workstation liquid handler to evenly transfer the transfection cocktails, many human errors, including inconsistent cell densities among the wells and uneven transfections, were eliminated, resulting in a very consistent and sensitive screening system. Additionally, ECBDC's Molecular Devices ImageExpress Automated cell imaging and analysis system allowed for fast data processing of the transfections; the system captured the DAPI, GFP, and DsRed2 images of the 17 transfected plates of 931 human miRNAs. ImageExpress further used the Metamorph analysis software to quantify the total fluorescence of DsRed2 and GFP gene expression for each transfected well. The imaged

raw data and quantified data analysis correlated with one another, suggesting that the screening system is consistent and sensitive and the data are reliable for the narrowing down of the large library to a smaller and focused miRNA pool. Overall, the system is very efficient and with the initial set-up and protocols being established, the screening of the large miRNA library against 3'UTR of any desired gene can be accomplished just within a few weeks.

Oct4 stem cell transcription factor was used as the initial target gene for the newly developed miRNA screening system. The objective was to screen the miRNA library for potential miRNA(s) that inhibits Oct4 gene expression by targeting its 3'UTR. It was hypothesized that, besides the already known miR-145 (Xu et al., 2009), at least one new miRNA from a library of 931 miRNAs will be identified to downregulate Oct4 gene expression by complementary binding to its 3'UTR. It was predicted that if a miRNA can downregulate Oct4-3'UTR expression, then based on the DsRed2 fluorescence images, its DsRed2 protein expression would be significantly decreased when compared to the highest DsRed2 expressions of only the DsRed2-Oct4-3'UTR transfections at the optimal concentration of 8.0 ng and similar to the lowest DsRed2 expression intensities from the 0.5 ng DsRed2-Oct4-3'UTR only transfections. Quantitatively, if a miRNA is a candidate against Oct4-3'UTR, then it should have equal to or more than 50% DsRed protein inhibition. From the imaged and quantitative results of the screening of miRNAs against Oct4-3'UTR, miR-4310 and miR-1253 showed significant decreased in DsRed2 expression intensities in the presence of upregulated miRNA transcripts with 76.26% and 58.21% DsRed2 inhibition, respectively (**Fig. 7** & **Table 1**). Western blotting further confirmed the inhibitory effects of miR-4310 and miR-1253 on the 3'UTR of Oct4 through the downregulation of pCMV-HA protein expression (**Figure 9c,d).** If these two miRNAs are further confirmed to be regulators of Oct4 gene expression by binding to its 3'UTR, then the system has successfully identified at least two novel miRNAs with significant functions in the CSC development mechanism and more specifically, in the Oct4 gene regulation pathway. Potentially, these newly found miRNAs could be used as biomarkers, targets, or treatment strategies for cancer prevention.

**4.2. Potential problems and limitations to the novel miRNA screening system**

Even though the screening system's methods and protocols were successfully established, there were many complications during the initial experimental set-ups and execution of the screening itself. The novel screening system's problems and limitations are discussed as follows:

1) *Cell contamination*. Since the cells were seeded on such a large scale for mass transfections, there were more chances for the cells to be contaminated with bacteria or fungus if careful antiseptic precautions were not taken during the cell seeding. There were also instances where the cells were contaminated after transfection. This was most likely due to the amount of steps in the transfection protocol. Each transfer of the plasmids, transfection media, and transfection cocktails by the MultiDrop dispenser and Sciclcone Workstation increases the chances of contamination due to increased contacts with the cultured cells. Contamination was successfully avoided for the two sets of mass transfections due to extra precaution measures, such as spraying down the equipment with 70% ethanol between each transfer of cells, plasmids, media, and transfection cocktails and autoclaving the instruments used for transfection.

2) *DsRed2 signals were not stable*. Another problem of the system was that sometimes there is an edge effect in the DsRed2 images, but not in GFP; in other words, the wells on the edges of the 96-well plates consistently show higher intensity in DsRed2 images and reads in comparison to the inner wells. These edge effects were consistently seen in all plates in the same transfection patch, but not seen in plates of the same transfection at a different time. This position effect might be due to evaporation of culture media during the 72 hours incubation period, the stacking and pressure of the plates on top of another, and/or the uneven transfer of the transfection cocktails to the outer wells in comparison to the inner wells by the transfer equipment. To avoid the possibility of edge effects, the wells on the edges in 96-well plates were not used during the mass transfections. However, even with the exclusion of the outer wells, the edge effect phenomenon in transfected plates cannot be completely avoided for every transfection. Hence, another measure was taken to avoid this effect, which is to spread the plates out individually in the

incubator rather than stacking them on top of one another. Both of these measures seem to reduce the edge effects in the DsRed2 intensity patterns in the transfected plates thus far.

Studies have shown that DsRed2, among many other RFPs, are cytotoxic when used with standard high-level expression systems (Strack et al., 2008). This gives another possible explanation to why an edge effect is seen for the DsRed2 expression, but not the GFP expression images. Therefore, in future screening experiments, cyan fluorescent protein (CFP) or yellow fluorescent protein (YFP) will be used, instead, to make CFP-3'UTR or YFP-3'UTR constructs of Oct4 or other desired genes in order to detect potential miRNA downregulators of these genes' 3'UTRs. Both CFP and YFP have shown to maintain stable fluorescence expression in adult stem cells with no evidence of cytotoxicity (Taghizadeh & Sherley, 2008); therefore, using either CFP or YFP in future screenings will make the system more stable and the data more reliable for narrowing down the miRNA pool to key miRNAs that can negatively downregulation the desired gene's expression by directly targeting their 3'UTRs.

3) *This system missed some miRNAs with weak downregulation effects against target genes.* Since this system selects candidate miRNAs based on fluorescence signals, which directly correlate to absence or presence of downregulation effects of miRNAs on 3'UTRs of target genes, some miRNAs with weak DsRed2 fluorescence signals, and thus, weak targeting effects, might be missed by this system. For example, the newly developed screening system did not detect any inhibitory effect of miR-145 on Oct4-3'UTR; this finding is contrary to the report that endogenous miR-145 and pre-miR mimic of miR-145 ("pri-miR-145) reduced the luciferase activity of wild-type Oct4 by 23% in Hela and human ESC cells (Xu et al., 2009). Although the minor effects of miR-145 on Oct4 and other stem cells transcriptions factors (Sox2, and Klf4) in ESCs could be detected by lucciferase activity assay, this system may not be sensitive enough to detect the reported 23% downregulation of miR-145 on Oct4-3'UTR. The system's lack of sensitivity in detecting miRNAs that have minimal effects on target gene's expression is a disadvantage of the system, but one could also argue that it is an advantage to such a large-scale screening. The obvious advantage is that the novel system can detect only the miRNAs with the most

regulatory significance, narrowing the present 931 miRNA library to a smaller pool with only significant downregulators.

4) *False positive candidates*. In some co-transfections of miRNA and DsRed2-Oct4-3'UTR, high DsRed2 % inhibitions are detected, as in the top 3 ranked inhibitors, miR-4315, miR-548i-3, and miR-548i-1 with DsRed2 % inhibitions of 89.72%, 82.48%, and 78.29%, respectively, but their direct downregulation on the pCMV-HA-Oct4-3'UTR protein expression were not detected in western blotting experiments (**Fig. 9c,d)**. The objective of this study is to identify the miRNAs that bind to complementary sequences of the 3'UTRs of Oct4 and inhibit protein synthesis by repressing translation or promoting mRNA degradation. However, these false miRNA positive candidates might affect DsRed2 protein expression indirectly through many other cellular processes aside from translation, such as replication or transcription. The experimental approach and methodology of this screening is based upon a well-documented assumption that most miRNAs regulate gene expression by decreasing translation of protein-coding genes through binding to 3'UTRs. Nevertheless, controversy still surrounds this proposed mechanism by which miRNA control gene expression. In these false positive candidates, miRNAs might universally downregulate other genes responsible for DsRed2 expression in vivo, but the true extent of this claim is not known.

### 4.3. Future plans

At present, we have successfully set-up the automatic miRNA screening system to individually screen 931 miRNAs in our current libary and identified two miRNAs that could target Oct4. We have learned how to improve the newly developed system based on the problems we faced during the initial set-up and screening experiments. As a reflection of this constantly improving screening system, our results further provide valuable information for future improvements, which include the setting up of better control parameters to make the imaged and quantitative data more reliable and stabilizing the fluorescence proteins' expressions. The next steps in this study are described as follows:

1) *Western blotting*. Perform western blot confirmations for the remaining narrowed down miRNAs.

2) *Perform luciferase activity assay*. Despite the western blot results further confirming the screening results that miR-4310 and miR-1253 are potential downregulators of Oct4 gene by targeting its 3'UTR, many other confirmation techniques and assays are necessary before the findings are confirmed, and one of which is a luciferase activity assay. Luciferase, which is a class of oxidative enzymes used in bioluminescence, is commonly used as a reporter to assess the transcriptional activity in cells that are transfected with a genetic construct containing the luciferase gene under the control of a promoter of interest.

3) *Generate a dose response curve of pDsRed2-Oct4-3'UTR*. The generation of a dose response curve of varying concentrations of the candidate miRNA against DsRed2-Oct4-3'UTR can further confirm the results found in this screening; if the miRNA has any effect on Oct4-3'UTR, then the DsRed2 expression will exponentially decrease with increasing miRNA concentrations and the DsRed2 expression will remain constant if the miRNA has no inhibitory effect on Oct4-3'UTR.

The goal of future experiments is to determine whether endogenous candidate miRNAs found through this screening system, such as miR-4310 and miR-1253, can also repress Oct4 gene expression in human embryonic stem cells (hESC). If the miRNA can downregulate Oct4 gene expression, then the decrease of Oct4 protein concentration should drive the hESC into differentiation, which is expected since Oct4 has been shown to function as a master switch during differentiation by regulating the pluripotent potentials of ESC. With the establishment of this novel screening system, Wang's lab also plans to screen for miRNAs that could regulate the expression of other interesting oncogene and tumor suppressors to facilitate other studies and projects. One future direction includes using the screening system to select key miRNAs that affect cell growth and transformation stature at the cell level. Then, through the collaboration with Dr. Roberd M. Bostick from the Epidemiology Department of Emory University, confirm the functions of these miRNAs at the human tissue level. Dr. Bostick's laboratory will compare the expression level of the miRNAs and their targets between human normal tissues and tumors.

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