

**ABSTRACT**

MicroRNAs (miRNAs) are a class of naturally occurring small non-coding RNAs that control gene expression to regulate a variety of developmental and physiological processes. The levels of miRNA expression can vary between different human cell lines and tissues. For example, miRNA-373 and miRNA-520c expression is not detected in MCF7 cells. Previous work has suggested that miRNA-373 and miRNA-520c promote tumour invasion and metastasis in MCF7 cells via expression through a retroviral vector (1). Here we present data for the expression of miRNA-373 and 520c in A549 and MCF7 cells using CompoZr™ Targeted Integration Technology. The CompoZr™ Targeted Integration Kit allows for efficient targeted integration using a well-validated pair of zinc finger nucleases (ZFN) designed to target the AAVS1 locus on human chromosome 19. An advantage of targeted integration is the elimination of the effects of genomic context on the expression of delivered transgenes. This technology allows for the expression of a user specified gene of interest and in this case miRNA genes.

**INTRODUCTION**

Zinc finger nucleases (ZFN) are fusions between zinc finger proteins and the non-specific nuclease domain of restriction enzyme FokI. Each zinc finger interacts with three nucleotides, and multiple (N) fingers can be assembled together to specifically bind a targeted sequence of 3N bases. FokI must dimerize to achieve double strand cleavage in the DNA. This means that a pair of ZFNs is required to bind and cut the targeted site (Figure 1). Specificity is determined by the number of fingers in the ZFNs. The ZFN is used to create a targeted double strand break that stimulates the cell's natural DNA repair processes of homologous recombination and Non-Homologous End Joining (NHEJ). These processes are harnessed to generate precisely targeted genomic edits, resulting in cell lines with targeted gene deletions, integrations, or modifications.

Co-transfection of a repair template (plasmid, etc) with the ZFN pairs allows for the integration of a gene of interest (GOI) into the genome via homologous recombination (HR; Figure 2). These ZFN pairs are expressed via a plasmid or in vitro transcribed mRNA. In this instance, we are using a well-validated pair of zinc finger nucleases (ZFN) designed to target the Adeno associated virus type 1 (AAVS1) locus on human chromosome 19. This is the only known AAVS1 integration site in the human genome. The site constitutively expresses a protein named p84 which is non-essential in most cell lines. With the use of plasmid pZDonor-AAVS1, which contains right and left homologous arms to the AAVS1 site, you can achieve site specific integration of your gene of interest by homologous recombination. An advantage of site specific targeted integration is the elimination of the effects of genomic context on the expression of delivered transgenes.

Here we present data for the expression of human microRNA genes miRNA-373 and 520c in A549 and MCF7 cells using CompoZr™ Targeted Integration Technology.

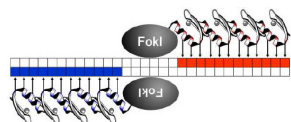


Figure 1. Schematic demonstration of a pair of four-finger ZFNs binding to a target DNA. Each of the two ZFNs binds to a 12 bp sequence on the opposite strand flanking the cleavage site. The two binding sequences are 6 nt apart. The FokI enzyme creates a double strand break that stimulates the cell's natural DNA repair processes of homologous recombination and Non-Homologous End Joining (NHEJ).

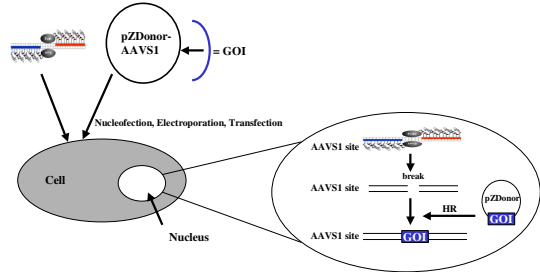


Figure 2. A ZFN pair targeting the AAVS1 site is co-delivered into a parental cell line with the pZDonor-AAVS1 plasmid containing a gene of interest (GOI), either by nucleofection, electroporation or transfection. The integration of the GOI occurs by homologous recombination (HR).

**MATERIALS**

- HeLa, A549 and MCF7 Cells
- CompoZr™ Targeted Integration Kit-AAVS1 (CTH-1KT; Sigma-Aldrich)
- Amnax Nucleofector II and Nucleofector® reagents (Amnax)
- QuickExtract DNA Extraction Solution (Epicentre)
- Jumpstart™ AccuTaq™ LA DNA Polymerase Mix (D5809; Sigma-Aldrich)
- TRI Reagent® (T9424; Sigma-Aldrich)
- Taqman® MicroRNA Assays (ABI)
- psiCHECK™.2 Reporter System and Dual-Luciferase® Reporter Assay System (Promega)
- Mission® microRNA Mimics (Sigma-Aldrich)

**RESULTS**

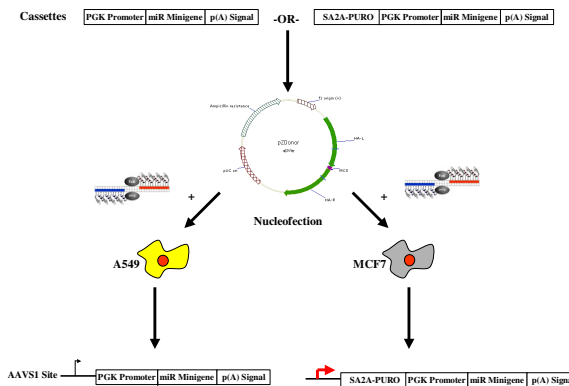


Figure 3. Cloning Strategy of miRNA Constructs. Cassettes generated for the expression of miRNA Minigenes in A549 and MCF7 cells. miRNA Minigenes were cloned from Human genomic DNA adapted from Voorhoeve et al. (2). This minigene contains the miRNA hairpin flanked by approximately 200 bases on each side. Each cassette contains a phage/lysate kinase (PGK) promoter, for miRNA Minigene expression, and a synthetic poly(A) signal. MicroRNA Minigenes cloned into the cassette were miRNA-373, miRNA-520c and tandem miRNA520c-373. All three combinations were used for A549 and miR-373 alone for MCF7. Also for MCF7 cells, a Splice Acceptor 2A fusion with a Puromycin selectable marker gene was inserted upstream of the PGK promoter. Puromycin selection was used due to lower homologous recombination activity in MCF7 cells when compared to A549 cells. Both cassettes were cloned into the pZDonor vector which contains right and left homologous arms to the AAVS1 site (HA-R and HA-L). One million cells were co-transfected with pZDonor plasmid and AAVS1-ZFN pairs (transcribed miRNA), via AMAXAS Nucleofector II. MCF7 cells were allowed to recover for 7 days and then selected for resistance in media containing 400-600ng/ml puromycin. The red arrow represents the p84 promoter that drives the puromycin gene expression. A549 cells were allowed to recover for 5 days, sub-cultured, and then allowed to grow for 7 more days. The transfected A549 cells were then sorted by single cell sorting into 96-well plates and then grown for 14-21 days. DNA was extracted from these cells via QuickExtract DNA Extraction Solution. These extracts were used in a junction PCR reaction (Jumpstart™ AccuTaq™ LA DNA Polymerase Mix) using oligonucleotide primers located within the AAVS1 genomic site and the cassettes PGK promoter (small arrows). This junction PCR was used to validate integration into the AAVS1 site.

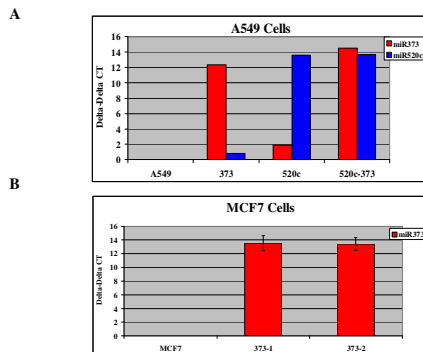


Figure 4. miRNA Expression from Integrated Constructs. In brief, total RNA was extracted from A549 and MCF7 cell clones using TRI Reagent. 100ng of Total RNA was used in a Taqman® MicroRNA Assays (ABI) with specific RT/Taqman probes for hsa-miR-373 (in red), hsa-miR-520c (in blue), and hsa-miR-21 (positive control and normanalog for Delta-Delta Ct). The Taqman® MicroRNA Assay is a two-step protocol with an initial RT step (16°C 30min., 42°C 30min., 85°C 5min., and a 4°C hold) followed by a qPCR (95°C 10min, 40 cycles of 95°C 15 sec., 60°C 60 sec.). The qPCR was performed on an Mx3000P thermocycler by Stratagene. The values plotted are Delta-Delta Ct's compared to parental A549 or MCF7 cells (done in triplicate for MCF7 clones). Single A549 clones (shown in A) express hsa-miR-373, hsa-miR-520c, or both. Two independent puromycin MCF7 clones (373-1 and 373-2) express hsa-miR-373 (shown in B).

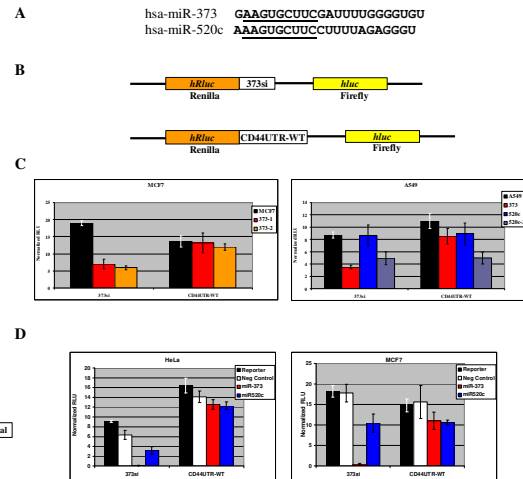


Figure 5. Function of miRNAs Expressed from Integrated Constructs. A. An alignment of hsa-miR-373 and 520c. Underlined is the conserved seed sequence. B. Dual-Luciferase Reporter constructs (Renilla and Firefly luciferases) were generated in the psiCHECK™.2 vector with an si target (373s) or CD44 target (CD44UTR-WT) cloned into the 3' untranslated region (UTR) of the Renilla reporter gene. The 373s1 target contains two 100% complementary sequences to the mature strand of hsa-miR-373 separated by 17 bases. The CD44UTR-WT target contains the last 1500 bases of the wild type UTR (~3100 bases) of human gene CD44, which contains a validated target for hsa-miR-373 and 520c. One hundred nanograms of 373s1 and CD44UTR-WT reporters were transfected into A549 and MCF7 clones (C) or co-transfected into HeLa and MCF7 cells with MISSION® microRNA mimics (D) via siPORT NeoFX. The final miRNA mimic concentration in D was 100nM for MISSION microRNA Mimic, Human miR-373 and 520c. Forty-eight hours post-transfection, cells were assayed using the Dual-Luciferase® Reporter Assay System. miRNA mimics in D were used to show that the validated CD44 target doesn't work well. Values plotted are normalized relative luciferase units (RLU) to the control firefly luciferase.

**CONCLUSIONS**

- Successful integration of microRNA genes into the AAVS1 site in A549 and MCF7 cells
- Expression of functional Human microRNAs 373 and 520c

**FUTURE DIRECTIONS**

- Express other Human miRNA genes and miRNA gene clusters
- Express miRNA Inhibitors
- Cell Migration
- SILAC (Stable isotope labelling with amino acids in cell culture)

**REFERENCES**

1. Huang, Q. et al. The microRNAs miR-373 and miR-520c promote tumour invasion and metastasis *Nature Cell Biology* 10, 202-210 (2008)
2. Voorhoeve et al. A genetic screen implicates miRNA-372 and miRNA373 as oncogenes in testicular germ cell tumors *Cell* 124, 1169-1181 (2006)

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