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Sequence-specific inhibition of microRNA-130a gene by CRISPR/Cas9 system in breast cancer cell line

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Abstract. MicroRNAs (miRNAs) are short stranded noncoding RNA that play important roles in apoptosis, cell survival, development and cell proliferation. However, gene expression control via small regulatory RNA, particularly miRNA in breast cancer is still less explored. Therefore, this project aims to develop an approach to target microRNA-130a using the Clustered Regularly Interspaced Short Palindromic Repeat (CRISPR)/Cas9 system in MCF7, breast cancer cell line. The 20 bp sequences target at stem loop, 3' and 5' end of miR130a were cloned into pSpCas9(BB)-2A-GFP (PX458) plasmid, and the positive clones were confirmed by sequencing. A total of 5 μg of PX458-miR130a was transfected to MCF7 using Lipofectamine® 3000 according to manufacturer's protocol. The transfected cells were maintained in the incubator at 37 ^{0}C under humidified 5% CO2. After 48 hours, cells were harvested and total RNA was extracted using miRNeasy Mini Kit (Qiagen). cDNAs were synthesised specific to miR-130a using TaqMan MicroRNA Reverse Transcription Kit (Applied Biosystems). Then, qRT-PCR was carried out using TaqMan Universal Master Mix (Applied Biosystems) to quantify the knockdown level of mature miRNAs in the cells. Result showed that miR-130a-5p was significantly downregulated in MCF7 cell line. However, no significant changes were observed for sequences targeting miR-130a-3p and stem loop. Thus, this study showed that the expression of miR-130a-5p was successfully down-regulated using CRISPR silencing system. This technique may be useful to manipulate the level of miRNA in various cell types to answer clinical questions at the molecular level.

1. Introduction

Breast cancer is the most common cancer among women worldwide. The mortality rates have significantly reduced in the past 20 years due to the implementation of screening programs and the development of new therapy [1]. Even though there are many findings in understanding the development and progression of breast cancer, only part of the molecular mechanisms underlying breast cancer pathogenesis is fully understood.

MicroRNAs (miRNAs) are short stranded noncoding RNA with 18-25 nucleotides in length, highly conserved in evolution and play important roles in almost all biological pathways [2]. miRNAs post-transcriptionally regulate the expression of protein-coding genes by binding to the specific binding sites in

their 3'UTR and causing either mRNA degradation or translational termination [2]. Briefly, primary-miRNA (pri-miRNA) is transcribed by RNA polymerase II [3]. In nuclease, enzymes Drosha and Pasha will recognize and cleaves pri-miRNA leaving two nucleotides overhangs in the 3'-end (3p) [4]. The precursor-miRNA (pre-miRNA, Figure 1) exit the nuclease and in the cytoplasm, Dicer will cleaves off the stem loop (SL) leaving 3'-end (3p) and 5'-end (5p) [5, 6]. One of the strand, usually 5p, will assemble into AGO to form RISC complex which will then cause translational repression [7]. The other strand will degrade rapidly [7].

In cancer, it has been demonstrated that dysregulation of miRNA can alter the synthesis of either oncogenic protein or tumor suppressor protein, which then leads to tumour formation and progression [8, 9]. miR-130a has been reported to be overexpressed or under-expressed in different types of cancer including colon cancer, non-small lung cancer, chronic myeloid leukaemia and hepatocellular carcinoma indicating the important roles of miR-130a in cancer development, progression and metastasis [8-11]. A study reported that miR130a is an important tumor suppressor in breast cancer by targeting RAB5A [12]. However, at present, there is a limited evidence of the role of miR-130a in oncogenesis of breast cancer.
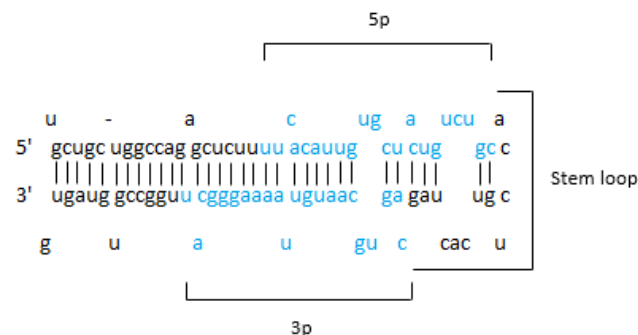

Figure 1. Figure shows the structure of pre-miR130a consisting of 5' (5p) end, stem loop and 3' (3p) end.

Thus, in order to investigate functional activity of miRNAs, selection of precise and efficient miRNA silencing technique is important. One of the genome editing technology, Clustered Regularly Interspaced Short Palindromic Repeats (CRISPRs) system is increasingly being employed to silence protein-coding genes in several model organisms [13]. CRISPRs are DNA loci with short repetitions of base sequences [14]. Short segments of "spacer DNA" are present in between each repetition from previous exposures to a virus [14]. Cas9 is a nuclease guided by small RNAs which promotes genome editing via double strand break at a targeted genomic locus [13]. Cas9 decreases the off-target effect as it is highly specific, efficient and well-suited for high throughput and multiple gene editing for different cell types and organisms [13, 15]. This double-nicking strategy using CRISPR-Cas9 approach has successfully generated stable knockout cell line models in human [16]. Thus, CRISPR system was used as a tool to silence miR130a in this study. This may then assist in studying the role of miR130a at molecular level in breast cancer.

In this study, the expression of miR130a-3p and miR130a-5p in different types of cell lines (human breast cancer cell line (MCF7), acute myeloid leukaemia cell lines (Kasumi-1 and SKNO-1), human normal breast cell line (MCF10A), human lung cancer cell line (A549) were compared adjacent to non-cancerous cell line (HEK293T) by real-time quantitative PCR. CRISPRs system was used to silence the expression of mature miR130a-3p and miR130a-5p in MCF7 cell line. The suppression activity was then confirmed by real-time quantitative PCR.

2. Materials and Methods

2.1 Cell and culture conditions
MCF7 was cultured in RPMI 1640 (Gibco, Thermo Fisher Inc, Grand Island, USA) medium supplemented with 10% heat inactivated fetal bovine serum (FBS) and 1% penicillin-streptomycin. MCF10A was cultured in DMEM (Gibco, Thermo Fisher Inc, Grand Island, USA) medium supplemented with 5% (v/v) horse serum, 0.2% EGF (20 mg/mL), 0.5 mg/mL hydrocortisone, 10 µg/mL insulin and 1% penicillin-

streptomycin. HEK293T and A549 were cultured in DMEM (Gibco, Thermo Fisher Inc, Grand Island, USA) medium supplemented with 10% heat inactivated FBS and 1% penicillin-streptomycin. Cultures were incubated in humidified atmosphere with 5% CO_2 at 37 ^{0}C.

2.2 Construction of plasmid miR130a-CRISPR

The oligonucleotides for site-specific chromatin cleavage of miR-130a target regions (Table 1) were designed using a bioinformatics filtering tool (http://crispr.mit.edu/). Oligonucleotides used to target site-specific chromatin cleavage were: miR130a-3p (3p) forward 5'-CACCGTGCAATGTTAAAA GGGCAT-3', reverse 5'-AAACATGCCCTTTTAACATTGCAC-5'; miR130a-5p (5p) forward 5'-CAC CGCACAATGTGAAAAGAGCTC-3', reverse 5'-AAACGAGCTCTTTTCACATTGTGC-3'; and miR130a-stem loop (SL) forward 5'-CACCGTGTAACACGATGACAGACG-3', reverse 5'-AAACCGTCTGTCATCGTGTTACAC-3'.

Each pair of oligonucleotide was phosphorylated and annealed at 37 °C (30 min) followed by 95 °C (5 min and then ramp down to 25 °C at 5 °C /min) in a thermal cycler (Bio-Rad MyCycler Thermal Cycler PCR). The annealed oligonucleotides were diluted at 1:200 with RNase-free water and cloned into the *BpiI* sites of pSpCas9(BB)-2A-GFP (Cp458) plasmid [13]. The plasmid was a gift from Feng Zhang (Addgene plasmid # 48138). The miR130a-CRISPR plasmids were then transformed into stable competent *E.coli* (NEB C040H) according to manufacturer's protocol. The transformed cells were spread on LB agar with ampicillin (100 µg/mL) and incubated overnight at 37 °C.

2.3 Screening oligonucleotide targets

A trace of two to three single colonies were picked and screened for oligonucleotide targets. PCR was carried out in a thermal cycler (Bio-Rad MyCycler Thermal Cycler PCR) using the following primers: forward 5'-GAGGGCCTATTTCCCATG-3'; reverse 5'-AAGGTCATGTACTGGGCACA-3'. The size (~600 bp) of each target was confirmed by running on 1% 1× TAE agarose gel according to the established protocol. The positive clones were then sequenced (1st BASE Sequencing, Selangor, Malaysia) to further confirm the targets for each clones.

2.4 Transfection

A total of 5 µg plasmids were transfected into 0.18×10^6 MCF7 cells by single transfection of 3p (miR130a-3p-Cp458), 5p (miR130a-5p-Cp458) or SL (miR130a-SL-Cp458) using Lipofectamine® 3000 (Life Technologies, Carlsbad, USA) according to manufacturer's protocol. Empty plasmid (Cp458) was used as a control. The transfected cells were maintained in the incubator at 37 ^{0}C under humidified 5% CO_2.

2.5 RNA isolation, reverse transcription (RT) and quantitative real-time PCR (RT-qPCR)

After 48 hours, cells were harvested and total RNA was extracted using miRNeasy Mini Kit (Qiagen, Germany) according to manufacturer's protocol. Reverse transcription and quantitative real-time PCR of mature miR130a-3p, miR130a-5p and small nucleolar U6 (RNU6B) normalisation control were performed as described [17, 18] with some minor changes. Briefly, 500 ng of total RNA was reverse transcribed by Taqman® microRNA reverse transcription kit (Applied Biosystems, Foster City, USA). Quantitative PCR (qPCR) was performed by Taqman® Universal Master Mix *II*, no UNG (Applied Biosystems, USA) using ABI StepOne™Plus Real-Time PCR Systems. The level of mature miR130a-3p (hsa-miR-130a-3p, Taqman® microRNA Assays) and miR130a-5p (has-miR-130a-5p, Taqman® microRNA Assays) expression was normalised to those of internal control (RNU6B, Taqman® microRNA Control Assays). The relative expression levels were evaluated using the $2^{-\Delta\Delta Ct}$.

2.6 Statistical analysis
Data was analysed using Microsoft Excel 2013. Statistical differences were determined by one tailed *t* test, with values of *p*< 0.05 considered statistically significant. Results were expressed as mean ± S.E.M. from minimum of three independent experiments.

3. Results

3.1 Screening on the expression miR130a-3p and miR130a-5p in different cell lines
The expression of miR130a-5p and miR130a-3p of HEK293T, MCF7 and MCF10A, were determined by RT-qPCR. Expression of miR130a-5p was significantly up-regulated in MCF7 (*p*< 0.01) when compared to HEK293T (Figure 2 A). Expression of miR130-3p was significantly up-regulated in MCF7 (*p*< 0.05) compared to MCF10A (Figure 2 B). HEK293T and MCF10A cell line were used as control for comparative CT method ($2^{-\Delta\Delta CT}$) expression analysis as they are considered as a non- or low-tumorigenic cell line. The results were presented as means ± S.E.M. from three independent experiments.
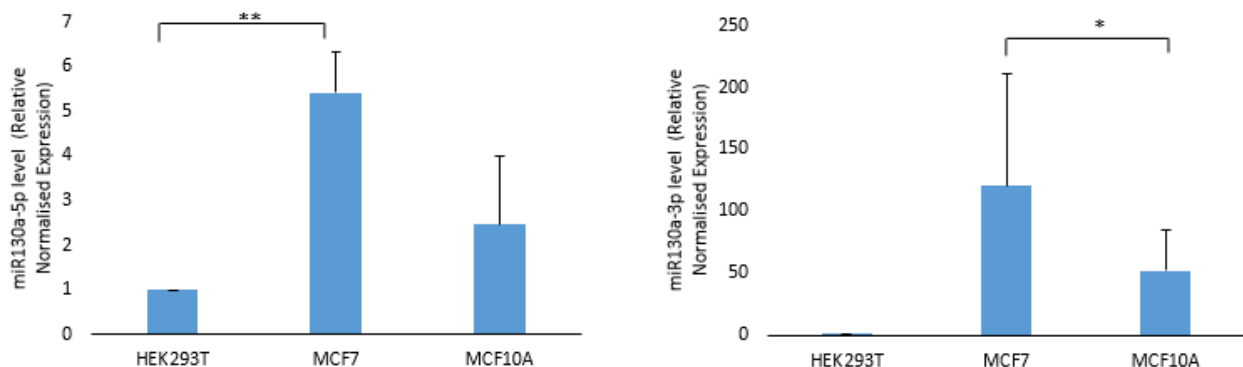

Figure 2. HEK293T, MCF7 and MCF10A expression levels of miR130a-5p (A) and miR130a-3p (B) detected by RT-qPCR (**p*< 0.05; ** *p*< 0.01 (one tail, paired *t* test)).

3.2 Analysis of single transfection
5p single transfection resulted in significant down-regulation of miR130a-5p by 2-fold compared to control. Both SL and 3p did not cause down-regulation of miR130a-5p expression (Figure 3 A). In contrast, single transfection of plasmid stem loop, 3p and 5p did not down-regulates the expression of miR130a-3p when compared to control (Figure 3 B). Transfection of empty Cp458 plasmid into MCF7 was used as a control for comparative CT method ($2^{-\Delta\Delta CT}$) expression analysis. Results were obtained from three independent biological replicates.
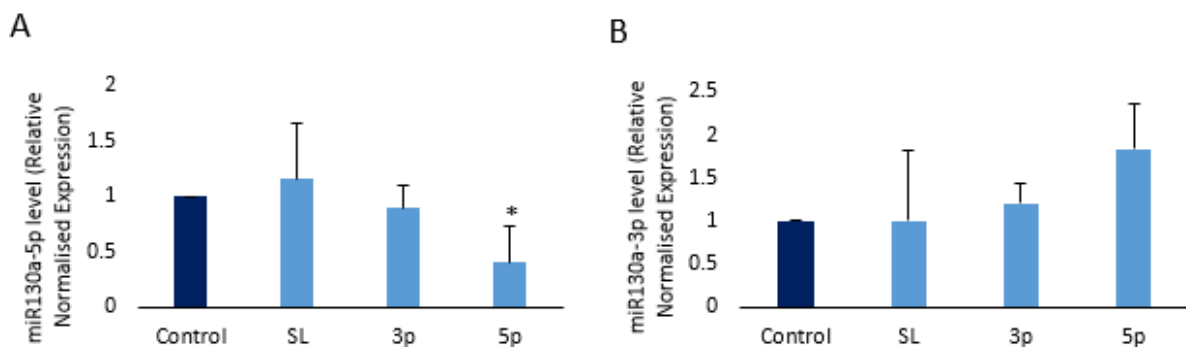

Figure 3. Down-regulation of the miR130a-5p (A) and miR130a-3p (B) expression by plasmid targeting 3p (miR130a-3p-Cp458), 5p (miR130a-5p-Cp458) or stem loop (miR130a-SL-Cp458) (**p*< 0.05 (one tail, paired *t* test)).

4. Discussion

miRNAs play important roles in the regulation of regulatory factors involved with the pathogenesis of cancer [19]. The changes in their expression is a common character of many cancers, including breast cancer [20]. In this study, miR130a expression analysis showed that the miR130a-5p was observed at highest level in MCF7 compared to HEK293 (p< 0.01). Expression of mir130a-3p was significantly higher in MCF7 compared to MCF10A (p< 0.05). The overall expression of miR130a-3p was higher compared to miR130a-5p in all cell lines. The reason behind this is that 3p remains as mature miRNA and therefore is preferentially integrated into the RISC [19]. As the expression of miR130a-5p and miR130a-3p was significantly high in MCF7, MCF7 was used as a model to down-regulate the expression of miR130a-3p and miR130a-5p by CRISPR/Cas9 system.

CRISPR/Cas9 systems has been shown to increase the efficacy of editing the genome differentiated in human cells [15, 21]. Thus, this system was used to down-regulate miR130a expression. The CRISPR/Cas9 system silenced either 3p, 5p or stem loop of the pre-miR130a in the cytoplasm. In the cytoplasm, pre-miR130a will be processed by the RNAse III enzyme Dicer producing mature miRNAs that are incorporated into a multi-protein complex [22]. Result shows that the expression of miR-130a-5p was significantly down-regulated with 5p single transfection. On the other hand, all single transfection does not cause significant change of miR130a-3p. This suggest that our CRISPR system works to suppress the mature level of miR130a-5p but not the miR130a-3p.

5. Conclusion

The expression of miR130a-5p was successfully down-regulated by single 5p transfection. This technique may be useful in answering certain clinical question at molecular level, such as role of miR130a in breast cancer cells.

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