ORIGINAL ARTICLE

Dose-and Time-Dependent Suppression of *Rac1* and *STIM1* in Acute Myeloid Leukaemia Cell Line Model

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ABSTRACT

Introduction: *Rac1* and *STIM1* genes are emerging therapeutic targets for cancers. However, their roles in acute myeloid leukaemia (AML) are not well understood. The goal of this study was to evaluate the effects of dose and time on *Rac1* and *STIM1* knockdown in the AML cell line model (THP-1 cells). **Methods:** THP-1 cells were transfected with si*Rac1* at doses of 50, 100, and 200 nM or dsi*STIM1* at doses of 2, 5, and 10 nM. Expression level of *Rac1* and *STIM1* then were assessed at time points between 12 and 72 h post-transfection using real-time reverse transcription polymerase chain reaction. **Results:** Compared to the control, 87% *Rac1* knockdown was attained with 50 nM siRac1 at 24 h post-transfection, and 70% *STIM1* knockdown was achieved with 10 nM dsiSTIM1 at 48 h post-transfection. **Conclusion:** These results show that effective knockdown of *Rac1* and *STIM1* is possible, and therapy that includes *Rac1* and *STIM1* inhibitors eventually could provide a new and highly effective strategy for AML treatment.

Keywords: Acute myeloid leukemia, *Rac1*, *STIM1*, siRNA gene silencing, Cancer therapeutic target

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INTRODUCTION

Acute myeloid leukaemia (AML) is a heterogeneous haematological cancer characterised by various genetic disorders, and it is associated with poor prognosis (10,17). Despite the improvement of outcomes in recent decades, 40–60% of patients with AML face relapse, 20% of patients do not respond to chemotherapy, and 5–10% of paediatric patients with AML die due to disease or chemotherapy complications (1,2). Gene silencing technology is considered to be an effective platform for studying cancer-induced molecular changes and identifying therapeutic targets. Recently, it also has been used as an alternative strategy for treating cancer.

Recent work on the Ras-related C3 botulinum toxin substrate 1 (*Rac1*) gene has shown that it plays a critical role in AML by increasing cell proliferation, migration, and resistance to chemotherapy (6,10). Stromal interaction molecule 1 (*STIM1*) is a component of store-operated calcium entry, and it also plays vital role in cancer cell proliferation, invasion, and migration (21-23).

High expression of *Rac1* and *STIM1* genes in some cancer tissues has been associated with poor patient prognosis, recurrence, and treatment failure (10,26,27). Recently there is increasing evidence suggests the presence of interaction between ROS and calcium signaling systems (28). In AML, the interaction between *Rac1* and *STIM1* remains unclear. The goal of this study was to evaluate the siRNA delivery and identify the optimal dose and time of *Rac1* and *STIM1* gene silencing in the AML cell line model (THP-1 cells). In the future, therapy that includes *Rac1* and *STIM1* inhibitors could provide a new and highly effective strategy for AML treatment.

MATERIALS AND METHODS

Cell Transfection

THP-1 cells were purchased from ATCC (Manassas, Virginia, USA) and were cultured in Dulbecco's modified eagle medium supplemented with 10% fetal bovine serum (Gibco, Life Technologies, Carlsbad,

CA, USA). Next, 1 x 10^6 THP-1 cells were transfected with *Rac1* siRNA (Stealth RNAi siRNA, Thermo Fisher, Carlsbad, CA, USA) using a Bio-Rad Gene Pulser Xcell electroporation system (Bio-Rad laboratories, Hercules, CA, USA) at pulse of 300 V for 7 microseconds. The transfected cells were diluted 20-fold with culture medium and incubated at 37°C and 5% CO2. Doses of 50, 100, and 200nM of *Rac1* siRNA were tested at 12, 24, 48, and 72 h post-transfection. *STIM1* dicer substrate siRNA (dsiRNA) (TriFECTa, Integrated DNA Technologies, Coralville, Iowa, USA) was transfected in to THP-1 cells at doses of 2, 5, and 10nM for 24 to 72 h post-transfection. Electroporated untreated cells (Ctrl) were used as the *Rac1* and *STIM1* expression controls.

To assess the efficiency of siRNA delivery into THP-1 cells, the cells were transfected with 10 nM fluorescein labeled dsiRNA, TYE 563 Transfection Control DsiRNA (TriFECTa, Integrated DNA Technologies, Coralville, Iowa, USA) using the same electroporation protocol. 24 h post-transfection, 8 x 104 cells/well were transferred to 96-well plate and washed with PBS then resuspended with 100µl PBS. After that, the cells were examined under the Olympus fluorescence microscope. Untransfected THP-1 cells were used as a control.

qRT-PCR Analysis

Total RNA was extracted from control and treated THP-1 cells at each time point using the RNeasy extraction kit (Qiagen, Hilden, Germany). A cDNA synthesis kit (Revere Tra Ace qPCR RT Master Mix, Toyobo, Osaka, Japan) and qPCR master mix kit (Luna universal qPCR master mix, New England Biolabs, Ipswich, Massachusetts, USA) were used to prepare the samples for gene expression assessment according to the manufacturers' instructions. The gene expression was assessed using Step One Plus real time PCR system (Applied Biosystems, Foster, CA, USA) with a set of *Rac1* primers (5'-GCCAATGTTATGGTAGAT-3' and 5'-GACTCACAAGGGAAAAGC-3') and *STIM1* primers (5'-AGAAACACACTCTTTGGCACC-3' and 5'-AATGCTGCTGTCACCTCG-3'). GAPDH was used as the endogenous control with primers 5'-AACGGATTTGGTCGTATTG-3' and 5'-GCTCCTGGAAGATGGTGAT-3'.

RESULTS

siRNA delivery into THP-1 cells

This work started with an assessment of siRNA delivery into THP-1 cells using fluorescent-labeled transfection control siRNA (TYE 563). The siRNA delivery does not affect THP-1 cells viability or phenotype as shown in Fig. 1 (A). A fluorescent microscope revealed that 80-85% efficient siRNA uptake into THP-1 cells 24 h post-transfection as shown in Fig. 1 (B).

Rac1 Knockdown Optimisation

After 24 h of siRac1 transfection into THP-1 cells, the three different doses (50, 100, and 200 nM) produced efficient *Rac1* mRNA suppression, which were 87%, 97%, and 95%, respectively, compared to control (Fig. 2). Among the tested time points, 24 h provided the most efficient *Rac1* suppression with a 99.8% reduction, while 12, 48, and 72 h revealed 2%, 65%, and 54%, respectively, *Rac1* mRNA suppression after transfection of cells with 50 nM si*Rac1* (Fig. 3).

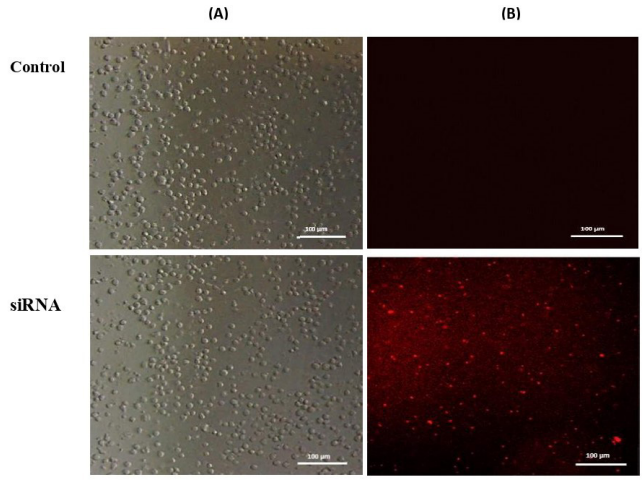

Figure 1: Fluorescent microscopy observation of THP-1 cells transfected with fluorescein-labeled siRNA 24 h post-transfection. (A) Bright-field microscopy of cells transfected with fluorescein-labeled siRNA in compares with control untransfected cells. (B) Fluorescent microscopy shows the red fluorescent signals inside the transfected cells indicates successful siRNA delivery into THP-1 cells. These findings were similar in the two repeated experiments

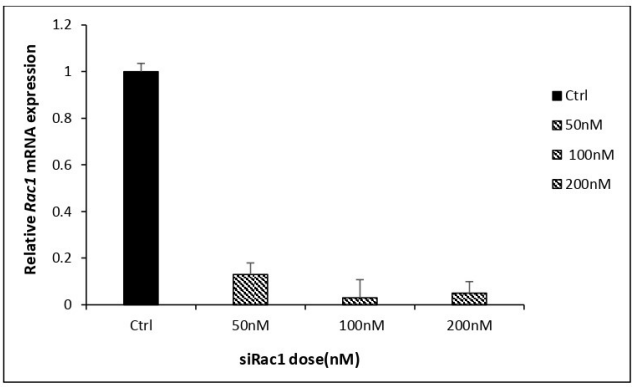

Figure 2: Dose-dependent *Rac1* knockdown at 50, 100, and 200 nM dose for 24 h transfection period. The error bar represents SD of three technical replicate data.

STIM1 Knockdown Optimisation

Transfection of THP-1 cells with 2, 5, and 10 nM dsi*STIM1* produced *STIM1* suppression with 44%, 4%, and 59%, respectively, at 24 h post-transfection (Fig. 4). The *STIM1* suppression rate reached to 70% at 48 h post-transfection of cells with 10 nM dsi*STIM1*, while at 24 h and 72 h, the suppression rate was 51% and 60%, respectively (Fig. 5).

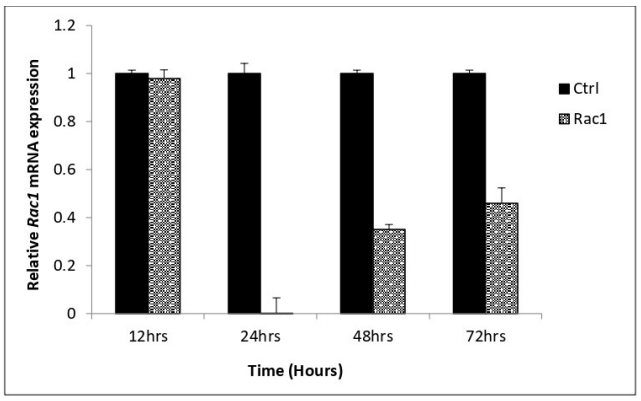

Figure 3: Time-dependent *Rac1* knockdown at 50 nM dose for 12, 24, 48 and 72 h. The error bar represents SD of three technical replicate data.

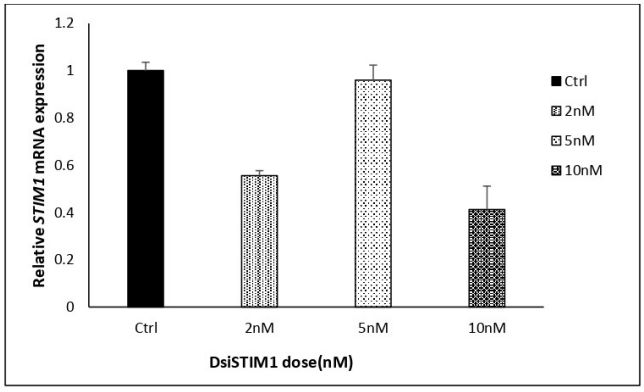

Figure 4: Dose-dependent *STIM1* knockdown at 2, 5, and 10 nM dose for 24 h transfection period. The error bar represents SD of three technical replicate data.

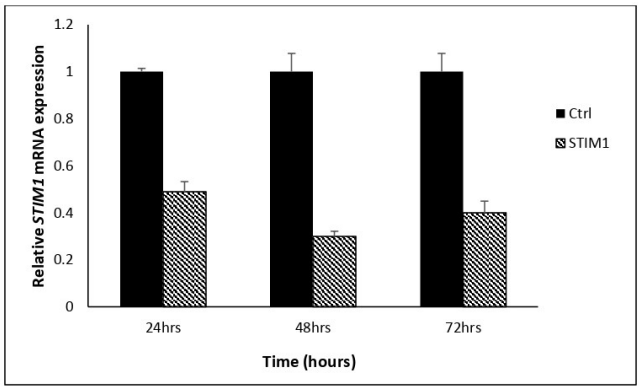

Figure 5: Time-dependent *STIM1* knockdown at 10 nM dose for 24, 48, and 72 h. The error bar represents SD of three technical replicate data.

THP-1 Cells Morphology Changes After *STIM1* Knockdown

Under bright-field microscopy, about 50-60% a decrease in the number of THP-1 cells was observed 24-48 h post-transfection with 10 nM dsiSTIM1 (Fig. 6). Other morphology changes included: cell aggregation, cell shrinkage, and apoptotic bodies also were observed at 24-48 h post-transfection (Fig 6).

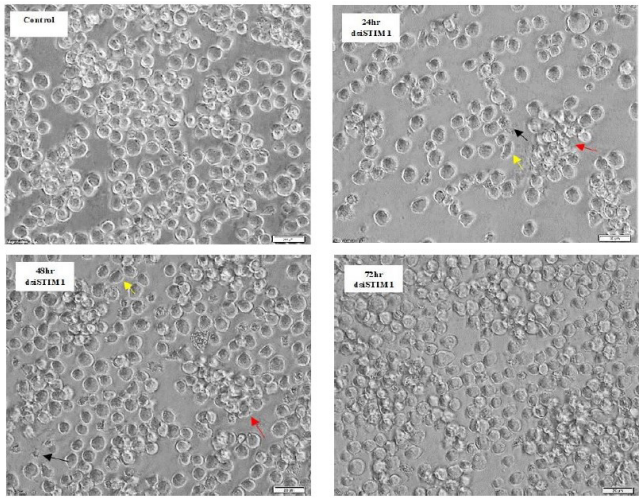

Figure 6: Preliminary morphological changes in THP-1 cells transfected with dsiSTIM1. The cells were transfected with 10 nM dsiSTIM1 for 24h, 48h, and 72 h periods. Under bright-field microscopy (magnification, 40x), decreased number of cells was clearly observed at 24-48 h. Cell aggregation (red arrow), shrinkage (yellow arrow) and apoptotic bodies (black arrow) were observed at 24-48 h. Similar observations showed in the biological replicate experiments.

DISCUSSION

The initial work started with quality control on siRNA transfection efficiency into THP-1 cells using fluorescent-labeled transfection control siRNA. Note that this study achieved a well-characterized transfection control where the successful siRNA delivery does not affect THP-1 cells viability or phenotype as shown in Fig. 1 (A). The success rate or efficient uptake of siRNA was monitored by visual evaluation under a fluorescent microscope using TYE 563 transfection control siRNA, which appeared as a red fluorescent signal inside the cells as shown in Fig. 1 (B). In this study, 80-85% efficient uptake was attained 24 h post-transfection. The transfection success and optimal siRNA uptake was reported to be above 70-80% (29, 30) with a threshold of 80% cell viability. It is important to assess the delivery success of siRNA as shown in this study before further functional analysis.

Further optimization work on dose-and time-dependent suppression in transfected THP-1 cells were achieved using siRac1 and dsiSTIM1. Transfection of THP-1 cells with siRac1 was profiled at 24 h with 50, 100, and 200 nM doses which exhibited an efficient *Rac1* mRNA suppression with 87%, 97%, and 95%, respectively, compared to control (Fig. 2). In knockdown experiments, the optimum dose for knockdown can be characterized if the targeted gene exhibits more than 65% suppression and selection of low effective dose is preferable to avoid off-target effect which may affect the results of further functional analysis (31). Therefore, THP-1 cells transfected with 50 nM siRac1 were chosen for further analysis on the time point suppression profile at 12,

24, 48, and 72 h. *Rac1* suppression rate reached the maximum level at 24 h with 99.8% reduction, while the other time points exhibited less *Rac1* mRNA suppression at 12, 48, and 72 h with 2%, 65%, and 54% respectively (Fig. 3). The optimum dose and time for knockdown are varying according to the cell line. In a study which has been conducted on HL-60 and KG1-a cell lines, the cells were transiently transfected with 30 nM siRac1 and they exhibited just 52% and 32%, respectively, reduction in the *Rac1* mRNA level at 48–72 h post-transfection (6). Our findings revealed that transient successful *Rac1* knockdown (>85%) was attained with 50 nM siRac1 after 24 h transfection.

The next evaluation was *STIM1* knockdown profile at 24 h post-transfection of THP-1 cells with 2, 5, and 10 nM dsi*STIM1* which produced *STIM1* suppression at 44%, 4%, and 59%, respectively, compared to control (Fig. 4). After that, the cells were transfected with 10 nM dsi*STIM1* and investigated at 24, 48, and 72 h time points to find that 70% was the highest reduction level of *STIM1* mRNA at 48 h post-transfection. At 24 and 72 h, *STIM1* reduction was 51% and 60%, respectively (Fig. 5). In a previous study included transfection of HL-60 cells with 1-3 µg siRNA specific to *STIM1* and STIM2, the knockdown was 80% and 60%, respectively, 36 h after transfection (7). Our results exhibited that *STIM1* was transiently successfully silenced (70%) with 10 nM dsiSTIM1 48h post-transfection.

Our work also revealed some changes in the morphology of the THP-1 cells after *STIM1* knockdown. Under bright-field microscopy, about 50-60% a decrease in the number of THP-1 cells was observed 24-48 h post-transfection with 10 nM dsiSTIM1 (Fig. 6). Other morphology changes included: cell aggregation, cell shrinkage, and apoptotic bodies also were observed at 24-48 h post-transfection (Fig 6). These morphological changes could possibly indicate the optimum dose and time for *STIM1* knockdown but, further evaluation still needs to ensure that these changes happened in correlation with efficient knockdown and if correspond with functional changes caused by *STIM1* silencing. For *Rac1*, no clear morphology changes were observed.

Successful dose and time optimization for *Rac1* and *STIM1* silencing was achieved in this study. Further functional and molecular studies are needed to investigate the potential role of *Rac1* and *STIM1* as therapeutic or prognostic targets for AML. Combined suppression of *Rac1* and *STIM1* could significantly improve the outcome among AML cases, especially in relapsed and chemoresistant patients.

CONCLUSION

In conclusion, this preliminary work identified the optimal dose and time for *Rac1* and *STIM1* knockdown in THP-1 cells and illustrated efficient *Rac1* and *STIM1* silencing in this AML cell line. Further molecular work is needed to investigate the potential role of *Rac1* and *STIM1* as therapeutic or prognostic targets for AML.

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