Small interfering RNA-mediated silencing of nicotinamide phosphoribosyltransferase (*NAMPT*) and lysosomal trafficking regulator (*LYST*) induce growth inhibition and apoptosis in human multiple myeloma cells: A preliminary study

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ABSTRACT

Multiple myeloma (MM) is a malignancy of B lymphocytes or plasma cells. Our array-based comparative genomic hybridization findings revealed chromosomal gains at 7q22.3 and 1q42.3, where nicotinamide (NAM) phosphoribosyltransferase (*NAMPT*) and lysosomal trafficking regulator (*LYST*) genes are localized, respectively. This led us to further study the functions of these genes in myeloma cells. *NAMPT* is a key enzyme involved in nicotinamide adenine dinucleotide salvage pathway, and it is frequently overexpressed in human cancers. In contrast, little is known about the function of *LYST* in cancer. The expression of *LYST* is shown to affect lysosomal size, granule size, and autophagy in human cells. In this study, the effects of small interfering RNA (siRNA)-mediated silencing of *NAMPT* and *LYST* on cell proliferation and apoptosis were evaluated in RPMI 8226 myeloma cells. Transfection efficiencies were determined by quantitative real time reverse transcriptase PCR. Cell proliferation was determined using MTT assay, while apoptosis was analyzed with flow cytometry using Annexin V-fluorescein isothiocyanate/propidium iodide assay. The NAMPT protein expression in siRNA-treated cells was estimated by enzyme-linked immunosorbent assay. Our results showed that *NAMPT* and *LYST* were successfully knockdown by siRNA transfection ($p < 0.05$). *NAMPT* or *LYST* gene silencing significantly inhibited cell proliferation and induced apoptosis in RPMI 8226 cells ($p < 0.05$). Silencing of *NAMPT* gene also decreased NAMPT protein levels ($p < 0.01$). Our study demonstrated that *NAMPT* and *LYST* play pivotal roles in the molecular pathogenesis of MM. This is the first report describing the possible functions of *LYST* in myelomagenesis and its potential role as a therapeutic target in MM.

KEY WORDS: Multiple myeloma; nicotinamide phosphoribosyltransferase; lysosomal trafficking regulator; small interfering RNA; cell proliferation; apoptosis

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INTRODUCTION

Multiple myeloma (MM) is a malignancy of B lymphocytes or plasma cells. It is a biologically complex disease characterized by excessive numbers of abnormal plasma cells in the bone marrow, and overproduction of intact monoclonal immunoglobulin of a single type [1]. It is the second most common hematologic cancer, representing 1% of all cancer diagnoses and 2% of all cancer deaths [2]. In Malaysia, more than 50% of myeloma patients are diagnosed at the late stage of the disease, and MM is more prevalent in men than women [3]. Gene expression changes, translocations, mutations, chromosomal deletions, and epigenetic changes are key factors underlying the molecular pathogenesis of MM [4-6]. Although recent advances in treatment strategies have improved survival and quality of life in patients with MM, MM is still an incurable disease. Drug resistance remains the main problem in MM patients.

Nicotinamide (NAM) phosphoribosyltransferase (NAMPT) is a key enzyme involved in nicotinamide adenine dinucleotide (NAD^+) salvage pathway [7]. Overexpression of NAMPT has been found in various human malignancies including ovarian, breast, colorectal, gastric, prostate, well-differentiated thyroid and endometrial carcinomas, and MM, melanoma,

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astrocytoma, and lymphoma [8]. The elevated expression of *NAMPT* gene promotes tumorigenesis through constant NAD resynthesis to provide adequate energy for rapidly proliferating cancer cells [9]. The inhibition of NAMPT is shown to induce cell death and reduce osteoclastogenesis in MM [10,11].

In contrast, little is known about the function of lysosomal trafficking regulator (LYST) in human cancer. The expression of *LYST* is shown to affect lysosomal size, granule size, and autophagy in human cells [12]. Mutation of *LYST* gene is associated with Chediak-Higashi syndrome (CHS), a rare autosomal recessive lysosomal disorder with hematological and immunological abnormalities [13]. Besides CHS, mutation in *LYST* gene is one of the key factors that cause hemophagocytic lymphohistiocytosis, a deficiency in immune system function, and life-threatening disease characterized by uncontrolled T-cell and macrophage activation [14]. In hemophagocytic lymphohistiocytosis, LYST plays an important role in controlling the terminal maturation of perforin-containing granules into secretory granules in cytotoxic T lymphocytes [15]. The role of *LYST* gene in oncogenesis is still unclear, and dysregulation of *LYST* gene has never been described in MM, nor other cancers, before.

Our previous array-based comparative genomic hybridization findings revealed gains at chromosomal 7q22.3 and 1q42.3 regions in 92% and 47% of Malaysian MM patients (n = 63), respectively [16]. More importantly, the *NAMPT* and *LYST* genes are located on 7q22.3 and 1q42.3, respectively. This led us to further study the functions of these genes in myeloma cell growth and survival by using small interfering RNA (siRNA) approach. Our findings provide a more profound understanding of the roles of *NAMPT* and *LYST* genes in the molecular pathogenesis of MM.

MATERIALS AND METHODS

Cell line

The myeloma cell line RPMI 8226 was purchased from the American Type Culture Collection (ATCC, USA). Cells were cultured in RPMI-1640 medium (ATCC) supplemented with 10% of fetal bovine serum (Lonza, Switzerland). All cells were cultured in a humidified incubator at 37°C containing 5% CO_2. The cells were passaged every 3-4 days.

siRNA transfection

Three unique siRNA duplexes for *NAMPT* (OriGene Cat. No.: SR306835, USA) and *LYST* (OriGene Cat. No.: SR300809, USA) were used to silence the respective gene in RPMI 8226 cells. The siRNAs were designated as NAMPT-a, NAMPT-b and NAMPT-c, and LYST-a, LYST-b and LYST-c. The siRNA sequences and their corresponding nucleotide binding sites are listed in Table 1. Approximately 200 nM of each siRNAs was used for transfection. Alternatively, pooled siRNAs were used (100 nM of each siRNA duplex). Pooled siRNAs were designated as NAMPT-abc and LYST-abc. Transfection of siRNAs into the RPMI 8226 myeloma cells was performed by Amaxa Nucleofection kit V (Lonza, Switzerland). Briefly, the cells were resuspended in 100 µl of nucleofector V solution mixed with 100-300 nM of siRNAs or scrambled negative control siRNAs or 2 µl of pmaxGFP at a density of 5.0×10^6 cells/mL. The mixture was transferred to a cuvette and nucleofected using G-016 pulsing parameter with an Amaxa nucleofector apparatus (Lonza, Switzerland). Then, the cells were immediately transferred to pre-warmed culture medium in 12-well plates. Transfection efficiencies were determined by quantitative real time reverse transcriptase PCR (RT-qPCR) at 24 and 48 hours post-transfection. Each transfection was performed in two replicates and in two independent experiments.

Total RNA extraction and first strand cDNA synthesis

Total RNAs were isolated from the cells according to the manufacturer's protocol (Qiagen miRNeasy mini kit, Germany). On-column DNA digestion was performed with

TABLE 1. siRNA sequences and their corresponding binding sites

siRNA	Sequences	Nucleotide binding sites and reference sequence accession number
NAMPT-a	AGAAUCUUAAGUUGGCUAAAUUCAA	2938-2962 (NM_005746.2)
NAMPT-b	GACAUACCCUAUAGAAUUACUAACC	2354-2378 (NM_005746.2)
NAMPT-c	AACAUGUAGUGAGAACAAUAAGCAT	3604-3628 (NM_005746.2)
LYST-a	GGCACAGACGAUGUGAAUAUUCUCA	1681-1705 (NM_000081.3)
LYST-b	GCAUGAAACCUAAUAUGUAUAUGTT	13351-13375 (NM_000081.3)
LYST-c	ACUGUUCAGAAGUCACAAAGAGGAG	3182-3206 (NM_000081.3)

siRNA: Small interfering RNA, NAMPT: Nicotinamide phosphoribosyltransferase, LYST: Lysosomal trafficking regulator

the RNase-free DNase set to eliminate DNA contamination during RNA purification (Qiagen DNase I, Germany). Five hundred nanograms of RNAs were reverse transcribed to cDNA in a final volume of 20 µl reaction containing ×1 reverse transcriptase buffer and enzyme mix (High capacity RNA-to-cDNA kit, Applied Biosystems, USA). The reaction was incubated at 37°C for 1 hour and stopped at 95°C for 10 minutes in a thermal cycler.

Quantitative real-time PCR (qPCR)

A pre-designed TaqMan probe was used to quantitate the *NAMPT* (Life Technologies Cat. No.: Hs00237184, USA) and *LYST* (Life Technologies Cat. No.: Hs00915897_m1, USA) gene expression. Each 20 µl reaction was prepared in triplicate containing TaqMan Gene Expression Master Mix (Life Technologies, USA), a FAM dye-labeled TaqMan Gene Expression Assay (Life Technologies, USA), and 50 ng of cDNA. Glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*) was used as endogenous control to normalize sample input and minimize the variation between the treated and untreated samples (Life Technologies Cat. No.: Hs02758991_g1, USA). All qPCR reactions were run on an ABI 7500 Fast Real-time PCR System (Applied Biosystems, USA) in 96-well format, and the thermal cycling conditions were 95°C for 20 seconds followed by 40 cycles of 95°C for 3 seconds, and 60°C for 30 seconds. All samples were normalized to the endogenous control and fold changes were calculated through relative quantification ($2^{-\Delta\Delta Ct}$).

3-(4,5-Dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium (MTT) assay for cell viability

The RPMI 8226 cells treated with siRNAs and scrambled negative control siRNAs were seeded onto 96-well plates at a density of 2.0×10^4/mL in triplicate (100 µl/well). The cell proliferation was evaluated at 24, 48, and 72 hours post-transfection. Briefly, 10 µl of MTT reagent (TREVIGEN, USA) at a concentration of 5 g/L was added to each well, and the cells were incubated for additional 4 hours at 37°C. Following incubation, 100 µl of detergent reagent was added to each well, and the cells were incubated at 37°C for 2 hours. After the insoluble crystals were completely dissolved, the plate was read at absorbance of 570 nm using Odyssey® SA Imaging System (Li-Cor, Lincoln, USA).

Annexin-V staining for cell apoptosis detection

Annexin-V staining was performed using the Annexin V-fluorescein isothiocyanate (FITC) apoptosis detection kit (BioVision, USA). In this assay, Annexin V-FITC (1:100) and propidium iodide (PI, 0.5 µg/mL) were used. Briefly, the cells treated with siRNAs and scrambled negative control siRNAs were collected by centrifugation. The cell pellet was washed with phosphate-buffered saline and resuspended in 500 µl of binding buffer. Annexin V-FITC (1:100) and PI (0.5 µg/mL) were added to the cell suspension and incubated for 10 minutes in the dark. Then, the cells were analyzed by FACSCalibur instrument (Becton Dickinson BD, USA). For each analysis, 10,000 events were recorded. Finally, the data generated were analyzed using the Cell Quest software (Becton Dickinson BD, USA).

Enzyme-linked immunosorbent assay (ELISA)

The cells transfected with siRNAs and scrambled negative control siRNAs were collected and lysed for 30 minutes on ice at 24 and 48 hours post-transfection. After centrifugation, supernatants were collected, and the total protein concentrations were measured by using the BCA assay kit (Thermo Scientific, USA). All the proteins were diluted to 500 µg/ml before applying the NAMPT Intracellular ELISA kit (BioVision, Switzerland). ELISA reactions were performed according to the manufacturer's instructions. Standard curve was generated using two-fold serial dilutions of the cells treated with control siRNA. All the samples and standards were loaded into the plate and incubated overnight at 4°C in duplicate. Following overnight incubation, the plate was washed and read at absorbance of 450 nm in an ELISA plate reader (TECAN Sunrise, Switzerland). The NAMPT protein concentrations were estimated from the standard curve equation.

Statistical analysis

Statistical analysis was performed using Student's *t*-test, and statistical significance was defined as $p < 0.05$. Data were expressed as mean ± standard deviation for two or three independent experiments.

RESULTS

Silencing of *NAMPT* and *LYST* with siRNA duplexes

In this study, pmaxGFP vectors were used to assess transfection success in RPMI 8226 myeloma cells. More than 80% of the cells expressed the green fluorescent signals at 24 hours post-transfection, indicating that the transfection was successfully procured (Figure 1A and B). The RT-qPCR results showed that the efficiency of *NAMPT* gene silencing was approximately 50% at 24 hours post-transfection when either NAMPT-a, NAMPT-b, or NAMPT-c siRNA was used (Figure 2A). The efficiency of gene knockdown decreased dramatically at 48 hours post-transfection for all three siRNA duplexes. When siRNA duplexes were pooled and

NAMPT-abc was used for the transfection, the gene knockdown efficiency was increased by up to >70% at 24 hours post-transfection (20% higher compared to the single siRNAs). The stability of the gene knockdown effect was maintained at 48 hours post-transfection when the pooled siRNAs were used (Figure 2A).

In addition, the RT-qPCR results showed that the efficiency of *LYST* gene silencing was >70% at 24 hours post-transfection when either siRNA duplex (LYST-a, LYST-b, LYST-c, or LYST-abc) was used (Figure 2B). Given that siRNA pooling is known to significantly reduce off-target effects and increase target specificity (especially in the case of *NAMPT* gene knockdown), pooled siRNAs, NAMPT-abc and LYST-abc, were used for the rest of the functional analysis.

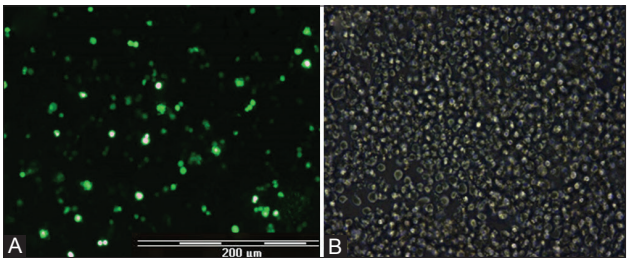

FIGURE 1. Estimated transfection efficiency. (A) RPMI 8226 cells were nucleofected with pmaxGFP vectors; the expression of green fluorescent protein was monitored at 24 hours post-transfection. (B) Image of the same cells under regular microscope with the same magnification as in A part.

siRNA-mediated silencing of *NAMPT* and *LYST* inhibited proliferation of RPMI 8226 cells

A significant decrease in the cell growth was observed in NAMPT-abc- and LYST-abc-treated cells when compared to the cells transfected with scrambled negative control siRNAs ($p < 0.05$) (Figures 3A and B). These findings showed that the suppression of *NAMPT* or *LYST* gene led to the inhibition of cell proliferation in RPMI 8226 cells.

NAMPT and LYST induced apoptosis in RPMI 8226 cells

To investigate the biological impact of *NAMPT* or *LYST* inhibition on the apoptosis in myeloma cells, RPMI 8226 cells were transiently transfected with NAMPT-abc or LYST-abc

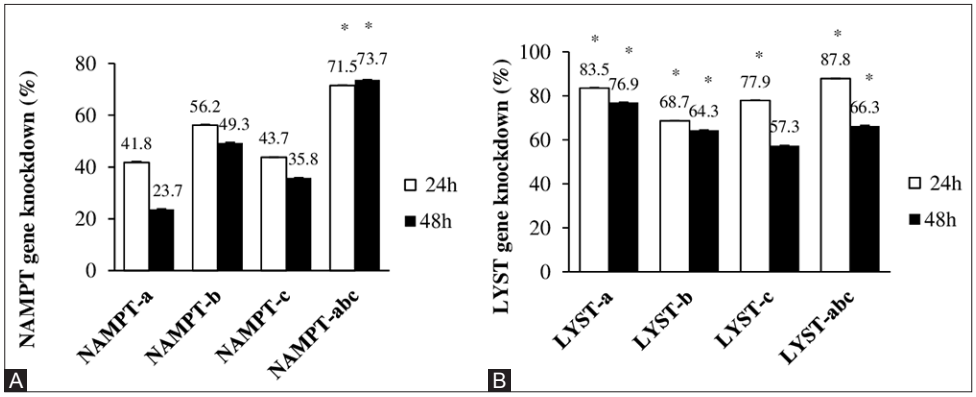

FIGURE 2. Effects of transfection with small interfering RNA (siRNA) on nicotinamide phosphoribosyl transferase (*NAMPT*) and lysosomal trafficking regulator (*LYST*) mRNA levels in RPMI 8226 cells at 24 and 48 hours post-transfection, determined by quantitative real time reverse transcriptase PCR. (A) Cells were transfected with either NAMPT-a, NAMPT-b, NAMPT-c (200 nM) or pooled siRNAs NAMPT-abc (100 nM for each siRNA). (B) Cells were transfected with either LYST-a, LYST-b, LYST-c (200 nM) or pooled siRNAs LYST-abc (100 nM for each siRNA). *$p < 0.05$ compared with the control.

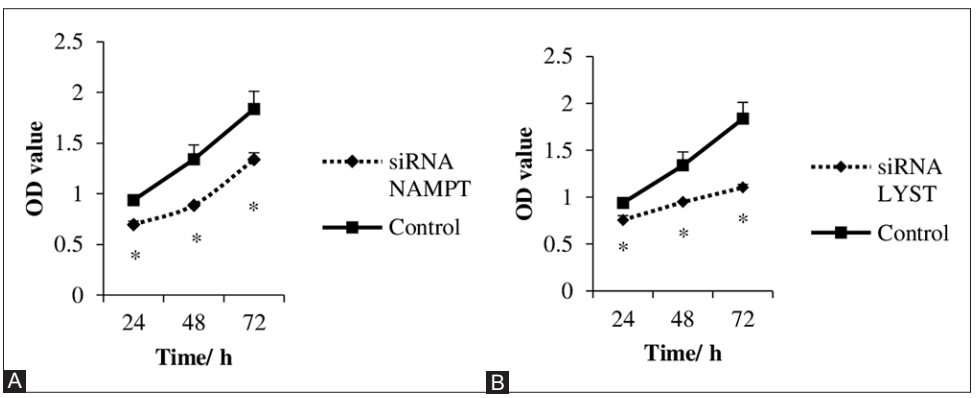

FIGURE 3. Proliferation of RPMI 8226 cells following transfection with nicotinamide phosphoribosyl transferase (NAMPT)-abc or lysosomal trafficking regulator (LYST)-abc siRNA for 24, 48 and 72 hours, determined by MTT assay. (A) Cell proliferation in NAMPT-abc-treated cells, (B) Cell proliferation in LYST-abc-treated cells. *$p < 0.05$ compared with the control.

siRNAs and the apoptotic cell death was measured using Annexin V-FITC/PI staining. The flow cytometry analysis showed that NAMPT-abc- and LYST-abc-treated cells significantly increased the numbers of early apoptotic cells compared to the cells treated with scrambled negative control, which was 26.9% versus 55.3% in NAMPT-abc-treated cells and 26.9% versus 41.5% in LYST-abc-treated cells ($p < 0.05$) (Figures 4A and B). This scenario indicated that silencing of *NAMPT* or *LYST* could induce apoptosis in RPMI 8226 myeloma cells.

Decreased protein expression level after the silencing of *NAMPT* gene

NAMPT protein expression levels in RPMI 8226 cells treated with siRNAs and scrambled negative control were estimated using ELISA assay. The OD readings indicated that the silencing of *NAMPT* gene using NAMPT-abc siRNAs resulted in a dramatic reduction of the NAMPT protein levels at 24, 48 and 72 hours post-transfection ($p < 0.01$) (Figure 5). Moreover, the ELISA assay showed that the protein expression levels were also reduced in NAMPT-b-treated cells, although the gene knockdown efficiencies for this siRNA were only about 50% at 24 and 48 hours post-transfection (Figure 2A and 5).

DISCUSSION

MM is a genomically complex heterogeneous disease composed of several molecular subtypes with varying clinicopathological features and disease outcomes [11]. This disease remains incurable although advances in treatment have improved the overall survival rate of the patients. Drug resistance is the major problem in MM therapy, which highlights the importance of identifying new molecular targets for therapies, to combat this disease [10].

The NAD^+ is a pivotal signaling molecule, which is involved in maintaining the functions of a wide variety of NAD^+-dependent enzymes in the cytoplasm and nucleus. NAMPT and nicotinamide mononucleotide adenylyltransferase (NMNAT) are two key enzymes included in the NAD^+ salvage pathway [17]. NAMPT catalyses the conversion of NAM to NAM mononucleotide (NMN), which is then converted to NAD^+ by NMNAT [18]. In humans, normal NAMPT expression is required during early embryo development, lymphocyte differentiation, muscle cell differentiation, maturation, and senescence [19-23]. In cancerous cells, more NAD^+ is needed to generate adenosine triphosphate, to supply energy for cell growth and survival [24]. Therefore, NAMPT expression is expected to be higher in cancer patients. The elevation of NAD establishes conditions for transcription regulation via

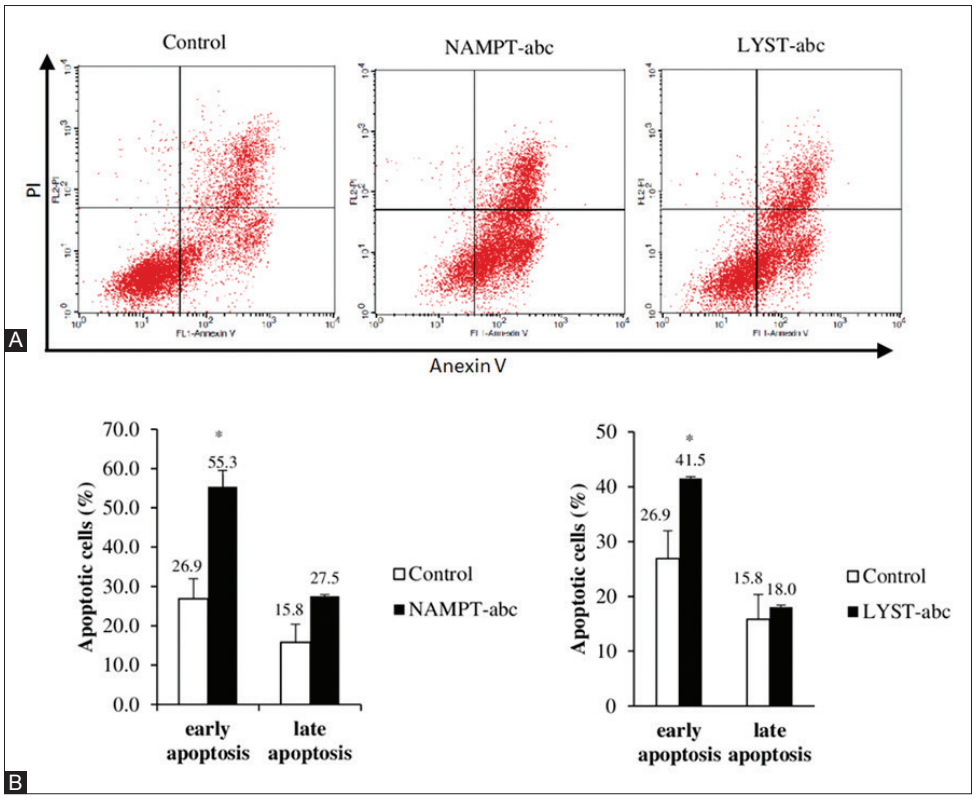

FIGURE 4. Apoptosis detection in RPMI 8226 cells with small interfering RNA (siRNA)-mediated silencing of nicotinamide phosphoribosyl transferase (*NAMPT*) or lysosomal trafficking regulator (*LYST*) gene at 48 hours post-transfection, analyzed by flow cytometry. (A) Scatter graphs show early and late apoptotic cells among RPMI 8226 cells treated with scrambled negative control, NAMPT-abc, and LYST-abc siRNAs. (B) Histograms show the numbers of early and late apoptotic cells among RPMI 8226 cells treated with NAMPT-abc and LYST-abc siRNAs compared to the cells treated with scrambled negative control. *$p < 0.05$ compared with the control.

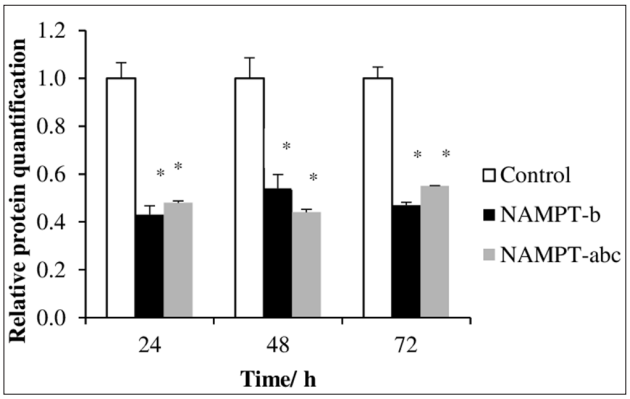

FIGURE 5. Nicotinamide phosphoribosyl transferase (NAMPT) protein expression levels in RPMI 8226 cells at 24, 48 and 72 hours following the transfection with NAMPT-b or NAMPT-abc small interfering RNA, estimated by enzyme-linked immunosorbent assay. *$p < 0.01$ compared with the control

two highly NAD^+-dependent enzymes: the protein deacetylase SIRT1 and poly-(ADP-ribose) polymerase (PARP1). Both SIRT1 and PARP1 play key roles in oncogenesis [18]. It has been suggested that SIRT1 regulates gene transcription and chromatin structure through transcription factors, co-regulators, and histone modifications. Tumor protein p53, nuclear factor κB (NFκB), FOXO (Forkhead box O) proteins, estrogen receptor α, liver X receptor, SRY-box 9 (SOX9), peroxisome proliferator-activated receptor gamma coactivator 1-alpha (PGC-1α), and p300 coactivator are all part of the critical downstream targets of SIRT1 [25-28]. Apart from that, PARP1 is involved in chromatin remodeling, transcription factor regulation, and DNA repair [29]. Examples of transcription factors regulated by PARP1 include octamer transcription factor 1 (Oct-1), specificity protein 1 (SP1), peroxisome proliferator-activated receptor γ (PPARγ), Smad3/Smad4, (sex determining region Y)-box 2 (SOX2), NFκB, and p300 [30]. Some of these transcription factors, such as p53, NFκB, FOXO, and p300 are involved in the MM development and progression [6,31-33].

In this study, siRNAs were transiently transfected into RPMI 8226 myeloma cells through nucleofection method. Our results showed that the gene knockdown efficiency and stability of the gene silencing effects were increased when the pooled siRNA fragments were used. Thus, we showed that pooled siRNAs could improve gene knockdown efficiency and stability of the gene silencing effects, in addition to the ability to reduce off-target effects [34]. Significant growth inhibition and apoptosis induction were detected in the siRNA-transfected cells demonstrating that *NAMPT* gene is a potential molecular target in myeloma therapy. More importantly, the reduction of NAMPT protein levels in NAMPT-b- and NAMPT-abc-treated cells suggests that the gene silencing had successfully suppressed the NAMPT translation in RPMI 8226 cells.

Recent studies have shown that the treatment of MM cell lines and xenograft models with FK866, a small molecule inhibitor, is able to inhibit NAMPT and induce MM cell death through autophagy mechanism. However, in these studies, the treatment of MM with FK866 did not induce cell death through apoptotic event [24,35]. The NAD^+ intracellular shortage, triggered by FK866 treatment, could cause autophagic cell death via two possible molecular mechanisms. First, autophagy is shown to be induced by inhibiting mammalian target of rapamycin signaling, a critical negative regulator of autophagy, along with a decrease in phosphoinositide 3-kinase and protein kinase B/AKT [35]. Second, the autophagy mechanism is induced by transcriptional activation of several autophagy-related genes through the inhibition of mitogen-activated protein kinase signaling pathway and nuclear localization of transcription factor EB [35]. Although these studies showed that MM cell death was induced by autophagy rather than apoptosis, it is possible that the pharmacological restriction of NAD^+ in MM cells triggers apoptotic signaling, which is then limited by concomitant onset of autophagy and the terminated apoptosis could then switch autophagy into MM cell death program. This explains why apoptosis cell death was not observed in the study of Cea et al. [35].

Unlike *NAMPT*, little is known about the function of *LYST* gene in human cancer. It has been shown that *LYST* gene is required for organizing endosomal resident proteins into late multivesicular endosomes by a mechanism that involves microtubules [36]. Recent findings have shown that *LYST* plays an important role in the transportation of materials into lysosomes, a place where toxic substances and bacteria are broken down and digested. Furthermore, *LYST* helps in maintaining the lysosomal size and regulating its movement within cells [12]. Our findings revealed that *LYST* gene was successfully knockdown by siRNAs in RPMI 8226 myeloma cells. For the first time, we showed that siRNA-mediated silencing of *LYST* inhibits proliferation and induces apoptosis in RPMI 8226 myeloma cells. The over-expression of *LYST* might contribute to the MM transformation and disease progression by activating cell proliferation and inhibiting apoptosis events.

Due to the budget constraint, the LYST protein expression level in the siRNA-transfected cells was not measured in the current study. Therefore, we are not certain whether *LYST* gene silencing has any impact on the protein translation. Since the ELISA kit for LYST protein detection is not commercially available, we aim to determine the effect of siRNA-mediated silencing of *LYST* on the protein expression by Western blot assay in our future research.

CONCLUSION

This study demonstrated that *NAMPT* and *LYST* genes play pivotal roles in the molecular pathogenesis of MM. This is the first report describing the possible functions of *LYST* in

myelomagenesis and its potential role as a therapeutic target in MM.

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DECLARATION OF INTERESTS

The authors declare no conflict of interests.

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