GENE EXPRESSION, BIOCHEMICAL AND FUNCTIONAL ANALYSIS OF STROMAL INTERACTION MOLECULE 1 (*STIM1*) SILENCING IN ACUTE MYELOID LEUKEMIA CELL LINES

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by

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I dedicate my thesis to

To the pure soul of my father and mother in paradise:

Salem Saeed and Salamah Mubarak Algariri,

My husband: Saleh Ahmed Alsobaai,

My mother-in-law: Munerah Saleh Alsobaai,

My kids: Abdullah and Salem,

All my friends and relatives.

Deep thanks for the love, doaa, sacrifice, and support

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LIST OF SYMBOLS

g	Gravitational force
kDa	Kilodalton
mg	Milligram
mg/ml	Milligram/ milliliter
ml	Milliliter
mM	Millimolar
nM	Nanomolar
nm	Nanometer
psi	Pound per square inch
UV	Ultraviolet
V	Volt
v/v	volume /volume
w/v	Weight/volume
µg	Microgram
µl	Microliter
µM	Micromolar
µs	Microsecond
µg/ml	Microgram/milliter
%	Percentage
°C	Degree Celsius

LIST OF ABBREVIATIONS

Akt	Protein kinase B
ALL	Acute lymphoblastic leukemia
AML	Acute myeloid leukemia
BAX	BCL2 Associated X
Bcl2	B-cell lymphoma 2
BM	Bone marrow
BSA	Bovine Serum Albumin
cDNA	Complimentary DNA
CMPs	Common myeloid progenitors
C-MYC	Avian Myelocytomatosis Viral Oncogene Homolog
CO2	Carbon dioxide
CR	Complete remission
CRC	Colorectal cancer
DNA	Deoxyribonucleic acid
DsiRNA	Dicer-substrate small interfering RNA
DUOX	dual oxidase
ERK	Extracellular signal-regulated kinase
FLT3	FMS-like tyrosine kinase 3

Fura-2AM	Fura-2-acetoxymethyl ester
GAPDH	Glyceraldehyde 3-Phosphate Dehydrogenase
H2DCFDA	2',7'-dichlorodihydrofluorescein diacetate
HIF-1α	Hypoxia-inducible factor 1-alpha
HSCs	Hematopoietic stem cells
IP_3R	Inositol 1,4,5-trisphosphate (IP3) receptor
KRAS	Kirsten Rat Sarcoma Viral Oncogene Homolog
LMPPs	Lymphoid-primed multipotent progenitors
MAPK	Mitogen-activated protein kinase
MCU	Mitochondrial Ca2+ uniporter
MCL-1	myeloid cell leukemia-1
MEPs	Megakaryocyte-erythrocyte progenitors
MM	Multiple myeloma
MPPs	Multipotent progenitors
mRNA	Messenger RNA
NADPH	Nicotinamide Adenine Dinucleotide Phosphate
NF-kB	Nuclear factor kappa B
NOXs	NADPH oxidases
Orai1	Calcium release-activated calcium channel protein 1
PBS	Phosphate buffer saline
PCR	Polymerase chain reaction
PI3K	Phosphatidylinositol-3-kinase
PKC	Protein kinase C

qRT-PCR	Quantitative reverse transcriptase polymerase chain reaction
Rac1	Ras-related C3 botulinum toxin substrate 1
RIPA	Radioimmunoprecipitation assay
RNA	Ribonucleic acid
ROS	Reactive Oxygen Species
RPMI	Roswell Park Memorial Institute Medium
RT	Reverse transcription
SDS-PAGE	SDS polyacrylamide gel electrophoresis
SOCE	Store-operated calcium entry
STIM1	Stromal interaction molecule 1
T-ALL	T cell acute lymphoblastic leukemia
TBS	Tris-buffered saline
TBST	Tris- buffered saline with TWEEN 20
TEMED	Tetramethylethylenediamine
Tg	Thapsigargin
TGS	Tris-Glycine-SDS
TRP	Transient receptor potential
WST-8	Water-soluble tetrazolium salt-8

LIST OF APPENDICES

EKSPRESI GEN, ANALISIS BIOKIMIA DAN FUNGSIAN PEMBUNGKAMAN MOLEKUL INTERAKSI STROMA 1 (*STIM1*) DALAM BARISAN SEL LEUKEMIA MIELOID AKUT

ABSTRAK

Molekul interaksi stroma (*STIM1*) baru-baru ini ditemui sebagai modulator kritikal pertumbuhan sel dan kelangsungan hidup dalam pelbagai jenis barah. Walau bagaimanapun, fungsi *STIM1* dalam AML masih belum difahami sepenuhnya. Oleh itu, tujuan kajian ini adalah untuk mengkaji kesan *STIM1* terhadap percambahan dan kelangsungan hidup garis sel AML dengan menggunakan teknik pembungkaman yang dimediasi oleh siRNA substrat dicer (dsiRNA) yang dijalankan pada dua garis sel AML, iaitu sel THP-1 dan Kasumi-1. Profil ekspresi gen kritikal terlibat dalam percambahan, kelangsungan hidup, dan pengeluaran ROS menganalisis melalui RT-qPCR selepas pembungkaman *STIM1*. Tahap kalsium dan ROS intrasel, percambahan sel dan pembentukan koloni juga mengukur selepas pembungkaman *STIM1*. Hasil kajian menunjukkan bahawa ekspresi mRNA *STIM1* lebih tinggi pada sel THP-1 berbanding sel Kasumi-1. Pembungkaman *STIM1* di tahap mRNA dan protein dicapai dalam sel THP-1 menggunakan 10 nM dsiSTIM1 selama 24 jam dan 20 nM dsiSTIM1 selama 48 jam dalam sel Kasumi-1. Pembungkaman *STIM1* menghasilkan tahap ekspresi gen *KRAS, MAPK, C-MYC, Akt, NOX2* dan *PKC* yang menurun dalam kedua-dua sel AML manakala tahap ekspresi gen *BAX* menaik dan *Bcl-2* menurun dalam sel THP-1. Selanjutnya, pembungkaman *STIM1* mengakibatkan pengurangan kadar kalsium dan ROS intrasel dan penghambatan percambahan sel AML dan pembentukan koloni pada kedua-dua sel AML. Sebagai kesimpulan, kajian ini menunjukkan bahawa *STIM1* mungkin memainkan

peranan penting dalam meggalakkan percambahan dan kelangsungan hidup sel AML. Ini mungkin melalui peningkatan gen yang berkaitan dengan proliferatif dan kelangsungan hidup, *KRAS, MAPK, C-MYC*, dan *Akt* dan juga boleh melalui kesan anti-apoptosis melalui gen *BAX* dan *Bcl-2*. Tambahan pula, *STIM1* mungkin memainkan peranan penting dalam mengekalkan tahap tinggi kalsium dan ROS intrasel yang mungkin menyumbang kepada kawalan laluan *KRAS* dan *Akt* yang dimediasi oleh *STIM1* serta peningkatan percambahan dan kelangsungan hidup AML. Kajian komprehensif lebih lanjut, termasuk analisis proteomik dan lebih banyak analisis fungsi mengenai peranan ketetapan *STIM1* bagi AML masih diperlukan untuk menyokong kajian ini dan menjadikan *STIM1* sebagai sasaran terapi yang menjanjikan harapan baik untuk AML.

GENE EXPRESSION, BIOCHEMICAL AND FUNCTIONAL ANALYSIS OF STROMAL INTERACTION MOLECULE 1 (*STIM1*) SILENCING IN ACUTE MYELOID LEUKEMIA CELL LINES

ABSTRACT

A stromal interaction molecule (*STIM1*) was recently discovered to be a critical modulator of cell growth and survival in a variety of cancers. In contrast, the function of *STIM1* in AML is still not properly understood. Therefore, the aim of the present study is to investigate the effect of *STIM1* on the proliferation and survival of AML cell lines by employing a dicer-substrate siRNA (dsiRNA)-mediated silencing technique performed on two AML cell lines, namely THP-1 and Kasumi-1 cells. The expression profile of critical genes involved in proliferation, survival, and ROS production was analyzed through RT-qPCR after *STIM1* silencing. The intracellular calcium and ROS levels, cell proliferation, and colony formation were also measured after silencing of *STIM1*. The study findings revealed that *STIM1* mRNA expression was higher in THP-1 cells compared to Kasumi-1 cells. *STIM1* silencing, at the mRNA and protein levels, was achieved in THP-1 cells using 10 nM dsiSTIM1 for 24 hours and 20 nM dsiSTIM1 for 48 hours in Kasumi-1 cells. *STIM1* silencing resulted in down-regulation of *KRAS, MAPK, C-MYC*, *Akt, NOX2* and *PKC* in both AML cells, whereas *BAX* was up-regulated, and *Bcl-2* was down-regulated only in THP-1 cells. Furthermore, *STIM1* silencing resulted in a reduction in the intracellular calcium and ROS levels and inhibition of AML cell proliferation and colony formation in both AML cells. In conclusion, the present study suggests that *STIM1* might have a crucial role in promoting the proliferation and survival of AML cells. This could be through enhancing the proliferative and survival pathway-related genes, *KRAS, MAPK,*

C-MYC, and *Akt,* and could also be through anti-apoptotic effects via BAX and Bcl-2 genes. Furthermore, *STIM1* could play a critical role in maintaining elevated levels of both intracellular calcium and ROS, which might contribute to *STIM1*-mediated control of *KRAS* and *Akt* pathways as well as the enhancement of AML proliferation and survival. Further comprehensive study, including proteomic and more functional analysis, on the regularity role of *STIM1* in AML is still needed to support the present work and to establish *STIM1* as a promising therapeutic target for AML.

CHAPTER 1

INTRODUCTION

1.1 Research Background

Acute myeloid leukemia (AML) is a malignant blood disorder described by the uncontrolled growth of abnormal myeloid progenitors in the bone marrow. AML accounts for about 1.1% of all new cancers cases and is responsible for around 1.9% of cancer deaths (National Cancer Institute, 2021). In the United States, the American Cancer Society reported 60,530 new cases of leukemia and 23,100 deaths from leukemia in 2020 and found 33% of leukemic cases were AML and 48% of leukemic deaths were due to AML. The overall AML 5-year relative survival rate is considered the worst (29.5%) compared to the other types of leukemia, which reached more than 70% (National Cancer Institute, 2021). Despite that, certain survival improvements, especially among the younger patients, have recently been observed (Sasaki et al., 2021).

AML survival has improved in line with advances in molecular characterization and therapy strategy of AML. Mutated FMS-like tyrosine kinase3/internal tandem duplication (FLT3/ITD) and Bcl-2 hyper-expression are some of the molecular disturbances that are found to play a role in AML survival (Pan et al., 2014). Currently, novel targeted therapies such as midostaurin, a tyrosine kinase inhibitor, and venetoclax, a Bcl-2 inhibitor, have been added to standard therapy and resulted in improving the complete remission (CR) rate to more than 50% in relapsed and refractory cases (Puccini et al., 2021; Wu et al., 2018). Since AML has a complex etiology, targeting multiple pathways could provide more benefits for AML cases (van Dijk et al., 2020).

Calcium ions are vital cellular molecules that are involved in numerous biological functions such as gene transcription, signal transduction, cell growth, and death (Prakriya, 2020). Growing evidence supports the contribution of disrupted calcium homeostasis to tumor initiation and progression (Gross et al., 2020). Store-operated calcium entry (SOCE), a store-dependent calcium channel, has been demonstrated to have a significant role in carcinogenesis. Stromal interaction molecule 1 (*STIM1)*, the main regulator of SOCE, was found to be dysregulated in many cancers, including multiple myeloma (MM), colorectal cancer (CRC), and oral cancer, where this dysregulation was reported to be associated with enhancing cell survival through regulation of certain vital oncogenes such as *Akt* (Singh et al., 2020; Wang et al., 2018; Wang et al., 2015).

Furthermore, the role of *STIM1* and SOCE in promoting melanoma and hepatocellular carcinoma cell proliferation and survival was explained to be through enhancing the *ERK* and *Akt* signaling pathways (Zhao et al., 2020; Umemura et al., 2014; Feldman et al., 2010). Recently, the involvement of the calcium/ROS interplay in carcinogenesis has been proven in a variety of malignancies through up-regulation of calcium and ROS-dependent proliferative and survival signaling pathways (*RAS/ERK, PI3K/Akt*) (Perillo et al., 2020; Feno et al., 2019; Takahashi et al., 2018). Therefore, SOCE is currently targeted with some chemical inhibitors such as SKF96365, 2-APB, and RP4010, and this inhibition results in suppression of cancer cell proliferation, colony formation, and migration through inhibition of Akt/mTOR, ERK1/2, and NFAT signaling (Singh et al., 2020; Anuj et al. 2020; Husain et al. 2020; Cui et al., 2017; Cheng et al., 2016; Huanyi et al. 2016).

The role of *STIM1* in AML is still not fully understood. Therefore, the current research targeted *STIM1* and evaluated its expression level in AML cell lines. The study also determined the effect of *STIM1* on AML cell proliferation and survival and on the expression of selected related genes through dicer-substrate siRNA (dsiRNA) mediated *STIM1* silencing. Furthermore, the research evaluated the proposed effect of *STIM1* on calcium and ROS levels. It is suspected that *STIM1* could play a significant role in promoting AML cell proliferation and survival through controlling proposed critical genes associated with the proliferative and survival pathways and through calcium/ROS interplay. The present work is suspected to provide new knowledge about the molecular and functional changes associated with AML, which, with further comprehensive work in the future, could contribute to discovering a promising therapeutic target for AML.

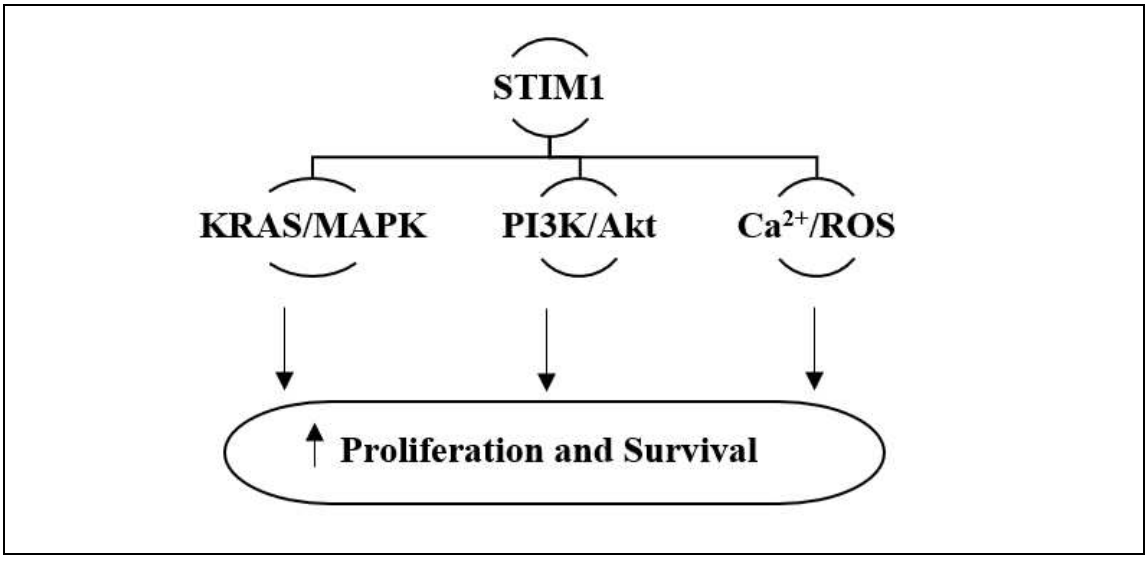

Figure 1.1 **Postulated effect of *STIM1* on AML cell proliferation and survival**. It is postulated that *STIM1* may promote the AML cell proliferation and survival through regulation of the proliferative *KRAS/MAPK* and survival *PI3K/Akt* signaling pathways and through controlling the calcium/ROS interplay.

1.2 Objectives of the study

General Objective:

The general objective of this study is to investigate the role of the stromal interaction molecule 1 (*STIM1*) gene in the proliferation and survival of AML cell lines.

Specific Objectives:

The specific objectives are:

1. To determine the optimal dicer-substrate small interfering RNA (dsiRNA)-mediated *STIM1* silencing in AML cell lines.
2. To investigate the effect of *STIM1* silencing on the expression of targeted genes involved in the proliferative, survival, and ROS pathways.
3. To assess the biochemical effect of *STIM1* silencing on the intracellular calcium and intracellular ROS levels in AML cell lines.
4. To evaluate the functional effect of *STIM1* silencing on the cell proliferation and survival (colony formation) of AML cell lines.

CHAPTER 2

LITERATURE REVIEW

2.1 Acute myeloid leukemia (AML)

2.1.1 Normal hematopoiesis and AML leukemogenesis

Normally, hematopoiesis is initiated by hematopoietic stem cells (HSCs), which form the starting point of the complex hierarchical hematopoietic system in the bone marrow (BM) (Wang & Wagers, 2011). HSCs are unique in their ability to self-renew throughout their lives as well as their ability to differentiate into multiple cell types. HSCs are divided into two types: long-term regenerating HSCs (LT-HSCs) and short-term regenerating HSCs (ST-HSCs). LT-HSCs can maintain their ability to self-renew and differentiate into multiple cell types throughout their lives. LT-HSCs can differentiate into ST-HSCs, which retain their multipotency but have a limited capacity for self-renewal. Furthermore, ST-HSCs differentiate into multipotent progenitors (MPPs) (Figure 2.1).

MPPs lost the ability to self-renewal but have the potential to differentiate into every functional blood cell type. MPPs differentiation gives rise to either lymphoid-primed multipotent progenitors (LMPPs) or common myeloid progenitors (CMPs). MPPs can also directly differentiate into megakaryocyte-erythrocyte progenitors (MEPs), which can also arise from CMPs. Common lymphoid progenitors (CLPs), which arise from LMPPs, together with granulocyte-macrophage progenitors (GMPs), which arise either from CMPs or LMPPs, in addition to MEPs, all differentiate into partially restricted progenitors that have the ability to produce all blood cell types (Cedar & Bergman, 2011; Wang & Wagers, 2011) (Figure 2.1).

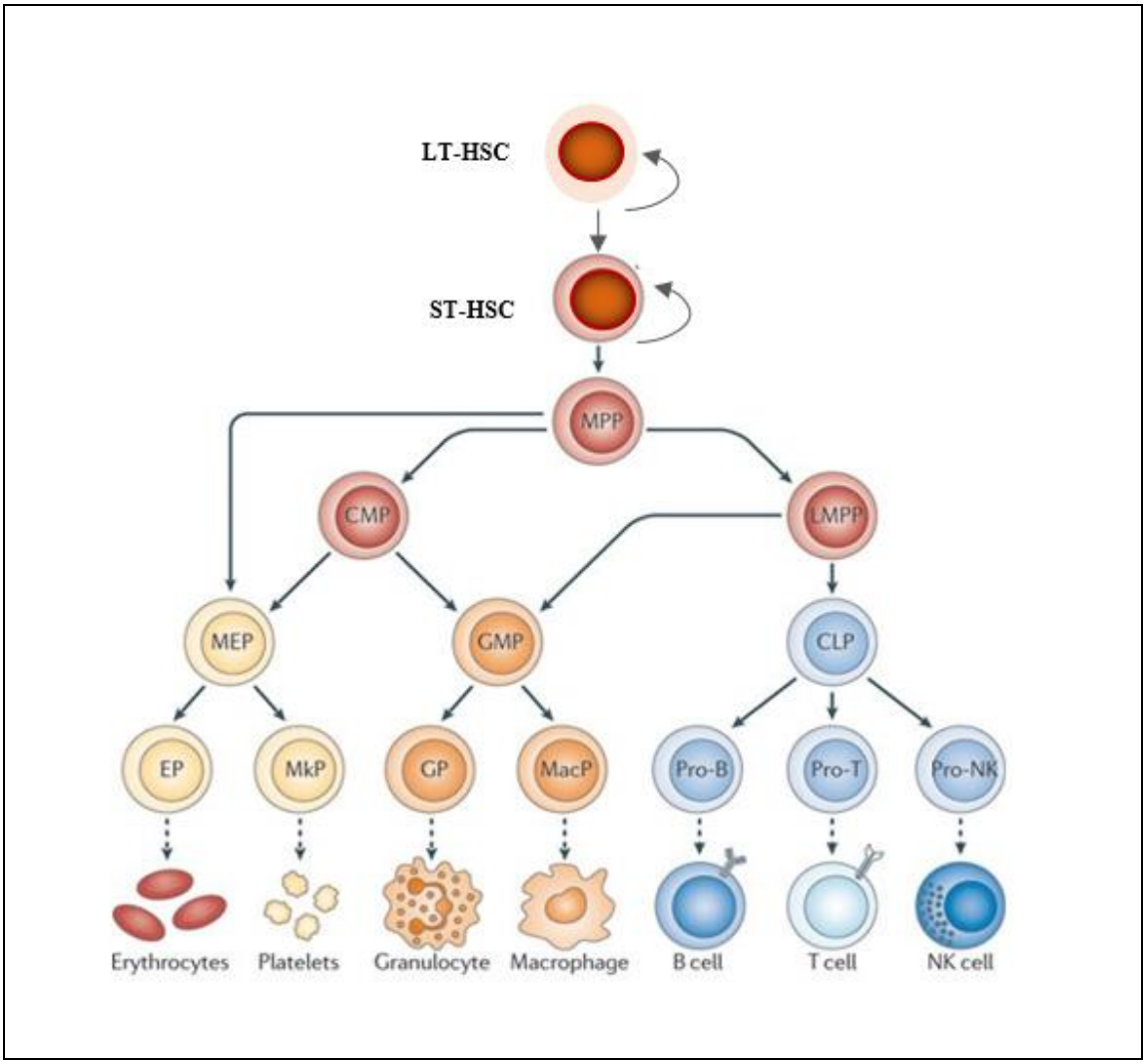

Figure 2.1 **Hierarchy of hematopoiesis.** Differentiation of HSC in an incremental manner. Progenitor cells select a specific line of the hematopoietic system and fail to differentiate into other lineages up till the cells reach the stage where progenitors can differentiate into only one lineage, such as erythrocyte progenitor (EP), megakaryocyte progenitor (MkP), granulocyte progenitor (GP), and macrophage progenitor (MacP). (Adapted from Cedar & Bergman. 2011).

Previously, the theory of the "two-hit hypothesis" explained the process of AML through the suggestion of the co-occurrence of two classes of mutations (Conway O'Brien et al., 2014). Class I mutations produce proliferative and/or survival benefits for the hematopoietic cells, such as fusion genes, mutant *RAS* or *FLT3*. Class II mutations reduce hematopoietic cell differentiation (Conway O'Brien et al., 2014). In addition to the Class I and II mutations, advances in AML research have exhibited the presence of epigenetic modifications in the AML genome that participate in the more complex AML process (Shih et al., 2012). Earlier, researchers reported a modified model for leukemogenesis where preleukemic mutations in landscaping genes such as *DNMT3A*, *IDH1*, and *IDH2* occur at the early stage of evolution and the transformation can happen later when the cells acquire additional proliferative mutations in genes involved in activated signaling such as *FLT3* and *KRAS* (Corces-Zimmerman et al., 2014) (Figure 2.2).

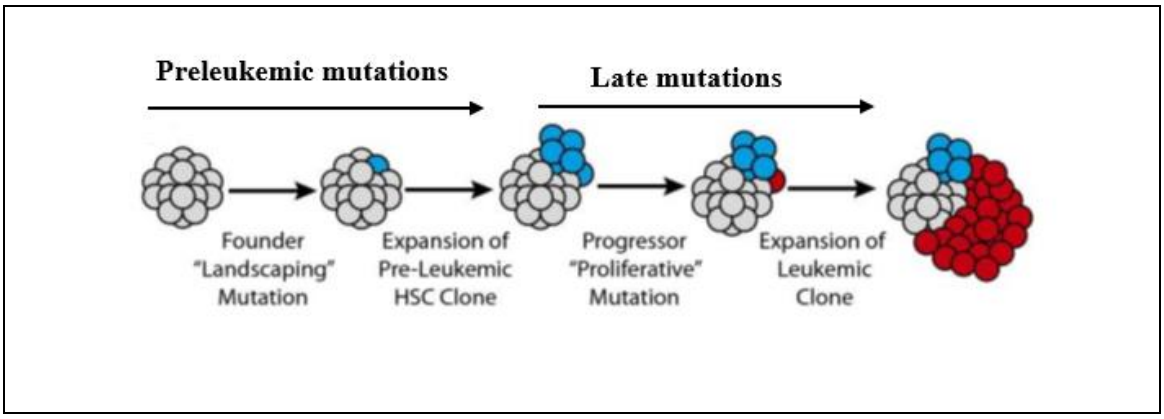

Figure 2.2 **Mutation acquisition model in AML**. Mutation acquisition in the AML evolution process starts with preleukemic landscaping mutations that involve epigenetic-modifying genes, followed by late proliferative mutations that occur in genes involved in signal transduction pathways and cellular proliferation. (Adapted from Corces-Zimmerman et al. 2014).

2.1.2 AML Epidemiology and classification

AML is a highly heterogeneous hematologic malignancy defined by the clonal expansion of immature (blast) myeloid precursors and disturbance of hematopoiesis in the bone marrow. In the last decade, the incidence of AML has increased worldwide and in Malaysia, as shown in Figure 2.3 (Global Health Data Exchange, 2019). Even though it affects people of all ages, adults are more likely to develop AML, with a median age of 67 and approximately 30% of AML patients over 75 (Chen et al., 2019). Pediatric AML is the fifth most frequent childhood cancer, and its prognosis is still poor compared to ALL, the most common type of leukemia in children (Chen et al., 2019). Recently, the AML survival rate revealed certain improvements which were prominent across all age groups except the age group older than 70 years.

From 2010 to 2017, the estimated 5-year survival rate of AML in the United States was 70% in childhood AML, 63% in patients less than 40 years old, 44% for patients aged 40-59 years old, and 22% for patients aged 60-69 years (Sasaki et al., 2021). The survival rate did not show any change among elderly AML patients where the survival rate remained just around 5% (Sasaki et al., 2021; Showel & Levis, 2014). Despite the higher AML survival rate among childhood AML, it is still less than the childhood ALL survival rate which reached 90% (Chen et al., 2019). There are many predisposing factors, also associated as risk factors, for AML, which include: 1) environmental factors as exposure to radiation, benzene, and tobacco smoke; 2) therapy-related factors such as radiotherapy and chemotherapeutic agents; 3) congenital disorders as Down and Bloom syndromes; and 4) hematological disorders like myelodysplastic syndrome, myelofibrosis, and aplastic anemia (Shallis et al., 2019).

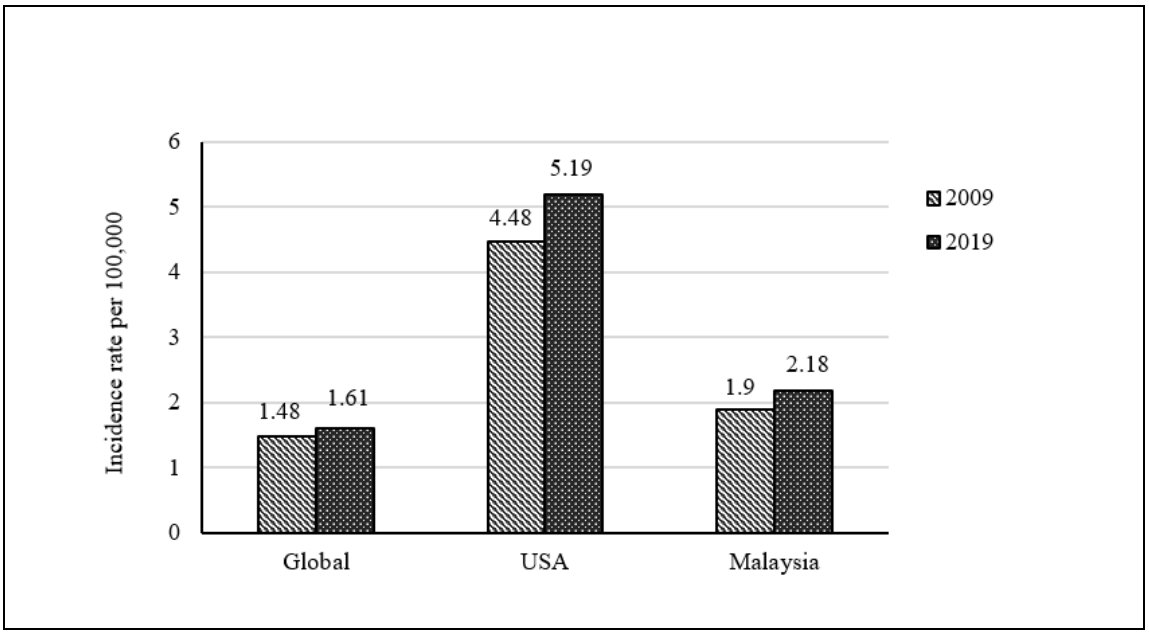

Figure 2.3 **AML incidence rate 2009-2019**. Comparison of AML incidence rate between 2009 and 2019 globally, in the USA, and in Malaysia. The data was obtained from the Global Health Data Exchange (2019).

The overcrowding of malignant myeloblasts in the bone marrow limits the potential for the development of normal red blood cells, white blood cells, and platelets, resulting in a reduction in their production and, clinically, anemia, bleeding, and frequent infections. The presence of ≥ 20% myeloblasts in the blood and bone marrow is considered a diagnostic finding for AML (Hwang, 2020). For diagnosis, therapeutic and prognostic purposes, AML was classified into subgroups according to two AML classification systems: 1) French-American-British (FAB) classification and 2) World Health Organization (WHO) classification. FAB classification is the initial AML classification system that was proposed in 1976 and classifies the AML according to the morphology and cytochemical staining of the blasts, which need to be 30% or more in the peripheral blood (Cheson et al., 1990; Segeren & Van't Veer, 1996). The FAB classification divided AML into 8 subtypes (M0-M7) (Table 2.1).

Table 2.1 FAB classification of AML

FAB Subtype	Name
M0	Acute myeloblastic leukemia, with minimal differentiation
M1	Acute myeloblastic leukemia, with minimal maturation
M2	Acute myeloblastic leukemia, with maturation
M3	Acute promyelocytic leukemia (APL)
M4	Acute myelomonocytic leukemia
M4eos	Acute myelomonocytic leukemia with eosinophilia
M5	Acute monocytic leukemia
M6	Acute erythroid leukemia
M7	Acute megakaryocytic leukemia

The World Health Organization (WHO) classification created its last revision, the 4th edition, in 2017 based on the update in genetic and molecular data on hematologic malignancies. It classified AML into six categories: 1) AML with recurrent genetic abnormalities 2) AML with myelodysplasia-related changes (MRC) 3) therapy-related myeloid neoplasms (t-MN) 4) AML, not otherwise specified (NOS) 5) myeloid sarcoma 6) myeloid proliferation related to Down syndrome (DS) (Table 2.2). According to this classification, in addition to the medical history and clinical information, AML diagnosis needs the results of morphological, immunophenotypic, cytogenetic, and molecular genetic testing. Assignment of AML to the recurrent genetic abnormalities category requires cytogenetic and molecular genetic testing (Hwang, 2020).

Table 2.2 **World Health Organization (WHO) classification of myeloid neoplasms and acute leukemia.** Revised 4th edition, 2017.

WHO classification of myeloid neoplasms and acute leukemia
Acute myeloid leukemia (AML) and related neoplasms
AML with recurrent genetic abnormalities AML with t(8;21)(q22q22.1); RUNX1-RUNX1T1 AML with inv(16)(p13.1q22) or t(16;16)(p13.1;q22); CBFB-MYH11 APL with PML-RARA AML with t(9;11)(p21.3;q23.3); KMT2A-MLLT3 AML with t(6;9)(p23;q34.1); DEK-NUP214 AML with inv(3)(q21.3q26.2) or t(3;3)(q21.3;q26.2); GATA2, MECOM AML (megakaryoblastic) with t(1;22)(p13.3;q13.1); RBM15-MKL1 Provisional entity: AML with BCR-ABL1 AML with mutated NPM1 AML with biallelic mutation of CEBPA Provisional entity: AML with mutated RUNX1
AML with myelodysplasia-related changes
Therapy-related myeloid neoplasms
AML, not otherwise specified (NOS) AML with minimal differentiation AML without maturation AML with maturation Acute myelomonocytic leukemia Acute monoblastic and monocytic leukemia Pure erythroid leukemia Acute megakaryoblastic leukemia Acute basophilic leukemia Acute panmyelosis with myelofibrosis
Myeloid sarcoma
Myeloid proliferations associated with Down syndrome Transient abnormal myelopoiesis (TAM) associated with Down syndrome Myeloid leukemia associated with Down syndrome

Both AML classifications, mainly WHO ones, are useful in providing a relatively accurate prediction about the treatment outcome of cancers. The correlation of FAB subtypes with the AML prognosis was previously investigated in patients who underwent BM transplants and the result revealed that M4-M6 were associated with lower overall survival and higher transplant-related mortality comparing to M1-M3 FAB subtypes (Fagioli et al., 1994). Currently, cytogenetic, and molecular status, in addition to patient age and health status, are considered powerful prognostic factors and may be therapeutic strategy guiding. In 2017, the European Leukemia Net (ELN) provided an updated risk stratification system which is dependent on cytogenetic and molecular aberrations to classify AML as favorable, intermediate, or adverse risk (Table 2.3) (Döhner et al., 2017).

Table 2.3 2017 ELN risk stratification.

Risk category	Genetic abnormality
Favorable	t(8;21)(q22;q22.1); RUNX1-RUNX1T1
	inv(16)(p13.1q22) or t(16;16)(p13.1;q22); CBFB-MYH11
	Mutated NPM1 without FLT3-ITD or with FLT3-ITD
	Biallelic mutated CEBPA
Intermediate	Mutated NPM1 and FLT3-ITD
	Wild-type NPM1 without FLT3-ITD
	t(9;11)(p21.3;q23.3); MLLT3-KMT2A
	Cytogenetic abnormalities not classified as favorable or adverse
Adverse	t(6;9)(p23;q34.1); DEK-NUP214
	t(v;11q23.3); KMT2A rearranged
	t(9;22)(q34.1;q11.2); BCR-ABL1
	inv(3)(q21.3q26.2) or t(3;3)(q21.3;q26.2); GATA2,MECOM(EVI1)
	25 or del(5q); 27; 217/abn(17p)
	Complex karyotype,§ monosomal karyotype
	Wild-type NPM1 and FLT3-ITD
	Mutated RUNX1
	Mutated ASXL1
	Mutated TP53

2.2 *STIM1* and Store-operated calcium entry (SOCE)

2.2.1 SOCE components

Calcium (Ca^{2+}) signals are considered vital cellular signals that control many cellular functions such as motility, transcription, cell division, growth, and apoptosis. Cellular Ca^{2+} homeostasis is preserved by numerous calcium pumps and channels such as voltage-gated channels (VGCC), ligand-gated channels (LGC), store-operated channels (SOC), transient receptor potential channels (TRP), and mechanically gated channels. Store-operated calcium entry (SOCE) is considered the main route of calcium entry in non-excitable cells and is activated following calcium store depletion in the endoplasmic reticulum. SOCE consists of two components: the endoplasmic reticulum (ER) part, which contains ER membrane located Ca^{2+} sensing STIM proteins, and the plasma membrane part, which is the oligomeric complex of Orai subunits.

STIM proteins, including STIM1 and its homologue, STIM2, have a similar overall structure with an N-terminal luminal domain and a C-terminal cytoplasmic domain. The luminal part of STIM1 consists of a canonical and non-canonical (hidden) EF-hand region connected to a sterile-α motif (SAM). The canonical EF-hand, a negatively charged region, binds Ca^{2+} and acts as a sensor for the luminal Ca^{2+} concentration, whereas the non-canonical EF-hand fixes the canonical EF-hand (Figure 2.4). The single trans-membrane TM domain of STIM1 connects the luminal with the cytoplasmic domain and offers structural flexibility to facilitate STIM1 conformational changes upon activation (Lunz et al., 2019; Rosado et al., 2016; Muik et al., 2012).

The cytoplasmic domain consists of three coiled-coil CC domains (CC1, CC2, and CC3), a CRAC modulatory domain (CMD), and a serine/proline- and a lysine-rich region (Figure 2.4). The CC1 domain consists of $CC1_{\alpha1}$, $CC1_{\alpha2}$, and $CC1_{\alpha3}$. Furthermore, the CC2 and CC3 domains consist of four regions, $S_{\alpha1}$, $S_{\alpha2}$, $S_{\alpha3}$, and $S_{\alpha4}$. The Orai-activating STIM1 fragments include the CRAC activation domain (CAD), the STIM Orai-activating region (SOAR), the Orai activating small fragment (OASF), and the coiled-coil domain-containing region b9 (Ccb9), which all contain CC2 and CC3 (Figure 2.4). The role of CC1 is mainly to keep STIM1 in a locked tight inactive conformation since ER-Ca^{2+} stores are full, whereas CC2 and CC3 domains are the functional domains that are responsible for activation of the Orai1 channel after binding of STIM1 to the PM by the K-rich C termini (Lunz et al., 2019; Rosado et al., 2016; Muik et al., 2012).

The Orai family includes Orai1, Orai2, and Orai3. The hexameric Orai channel complex has four transmembrane helices (TM1-TM4). In the Orai channel complex, the pore is in the center of the channel and is formed by the ring of TM1 helices, the first ring of the Orai subunits. TM2 and TM3 form the second ring, which is followed by the third ring that is formed by TM4. The Extended Transmembrane Orai1 N-terminal region (ETON) is the representative of the cytosolic TM1 proximal N-terminal part of Orai, which extends to reach the TM1 long into the cytosol. Likewise, TM2 and TM3 expand into the cytosol, but to the least extent. The cytosolic extensions of TM4 representing the C-termini reveal two different kink angles in a highly conserved hinge region, leading to an antiparallel dimeric interaction of the C-termini of two adjacent Orai molecules in the hexamer (Lunz et al., 2019; Rosado et al., 2016; Muik et al., 2012).

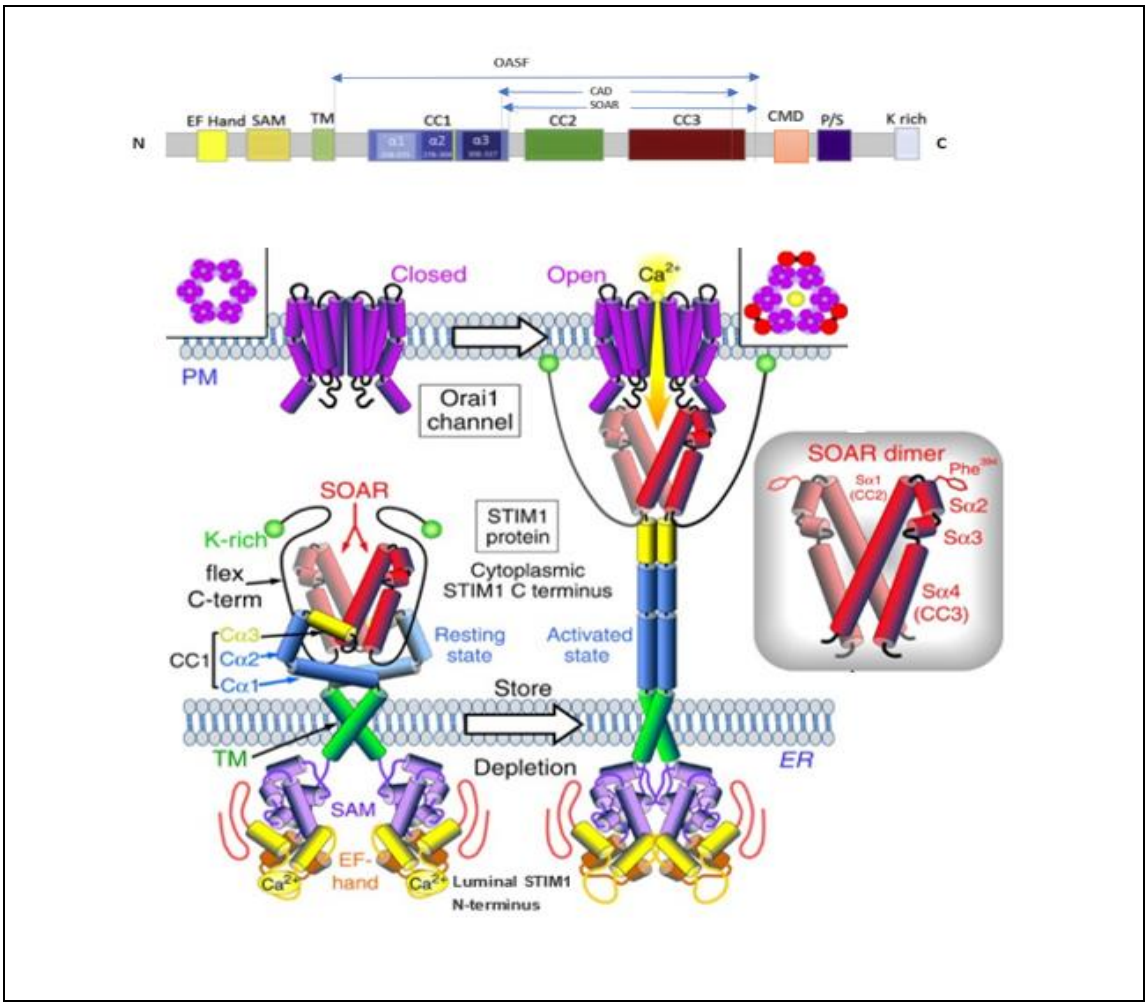

Figure 2.4 **Molecular models of STIM1 and Orai1 in resting and activated states.** The dimeric STIM1 protein senses Ca^{2+} changes via the EF-SAM domain. In the resting state, STIM1 is closed and folded. Depletion of calcium stores in ER resulted in activation of STIM1, which started with the conformational change of the EF-SAM region. The cytoplasmic STIM1 C terminus undergoes unfolding and elongation, and the K-rich C termini extend to bind the PM, which enhances the exposure of the SOAR region to initiate an interaction with Orai1. The hexameric Orai channel complex has four transmembrane helices (box, left). The SOAR/Orai1 binding leads to activation and opening of the channel and allows Ca^{2+} influx (box, top right). Each monomer of the SOAR dimer has four α-helices, $S_{\alpha1}$, $S_{\alpha2}$, $S_{\alpha3}$, and $S_{\alpha4}$ (inset, right). (Adapted from Lunz et al., 2019 and Zhou et al., 2015).

2.2.2 SOCE function

Store-operated calcium entry (SOCE) is the predominant channel of calcium entry in non-excitable cells and is considered part of a biphasic Ca^{2+} signaling system that includes both intracellular Ca^{2+} release and Ca^{2+} influx via transmembrane channels (Putney et al., 2017). Many non-excitable cells, such as neutrophils and macrophages, rely on SOCE for activation and function. In the hematological system, SOCE is critical for immunity, platelet function, and hemostasis. Antigen recognition by the T cell receptor (TCR) activates SOCE, which has been shown to influence a variety of cellular processes, including T cell growth, differentiation, death, and cytokine gene expression. SOCE is also essential for pathogen killing within the phagosomes of neutrophils and macrophages through calcium-mediated ROS production from NADPH oxidase (Bergmeier et al., 2013). SOCE also controls platelet function in hemostasis.

Platelets initiate hemostasis at the site of vascular injury and collaborate with the clotting system to stop bleeding and close the injured site. Research in this field supports the role of SOCE in the activation of platelets and plasma clotting factors and in the formation of a fibrin plug in the vascular injury area (Bergmeier et al., 2013). Activation of SOCE starts immediately following calcium store depletion in ER, which is mainly through the Inositol 1,4,5-trisphosphate (IP3) receptor (IP3R). Stimulation of IP3R could be through surface receptors such as TCR and chemokine receptors that activate phospholipase Cγ or Cβ, which then activate IP3, or internally through ROS-mediated activation of IP3R. In the resting state, STIM1 is kept in a closed and folded state through certain STIM1 TM domains conformation and an inhibitory clamp presented in the STIM1 cytoplasmic domain (Lunz et al., 2019; Muik et al., 2012).

SOCE activation following Ca^{2+} dissociation from the cEF hand stimulates a conformational change of the EF-SAM region. These structural changes establish signal transduction through the TM domain to the STIM1 cytoplasmic domain and enhance the exposure of the SOAR region to initiate an interaction with Orai1. The small CAD fragment directly binds to the Orai1 C-terminus and is considered the main interaction site between STIM1 and Orai1. Following STIM1-Orai1 coupling, Orai1 is activated. The activating signal is then transmitted from the Orai1 channel periphery across all four TM domains, eventually reaching the channel pore domain, which is formed by a ring of TM1 helices. Since the Orai1 channel was activated, the pore opened to allow the influx of Ca2+ from the extracellular space to the cytoplasm (Lunz et al., 2019) (Figure 2.4).

Recent studies have discovered that dysregulated calcium channels and homeostasis are significantly linked to several cellular processes implicated in the carcinogenesis of many cancers. SOCE components STIM1 and Orai1 were discovered to play important roles in cell proliferation, invasion, metastasis, and apoptosis escape in a variety of cancers (Bergmeier et al., 2013; Vashisht et al., 2015). The details about the function of STIM1 and SOCE in cancer cell growth and survival are discussed in the next section of this chapter that also explains their participation in keeping the interaction between calcium and ROS to maintain cancer cell survival.

2.3 *STIM1* and SOCE in Cancer

2.3.1 *STIM1* and SOCE in cancer cells proliferation and survival

The role of SOCE proteins, especially *STIM1* and *Orai1*, in cancer cell proliferation and survival has been well established in many cancers (Liu et al., 2020; Ge et al., 2019; Jardin & Rosado, 2016) (Figure 2.5). Yang et al (2009) provided the first evidence about the essential role played by SOCE protein, *STM1* and *Orai1*, for enhanced breast cancer migration and metastasis. Knockdown of *STIM1* and *Orai1* revealed a clear reduction in cell proliferation among certain glioblastoma cells (Vashisht et al., 2015; Li et al., 2013) and renal cell carcinoma (Kim et al., 2014). In non-small lung cell cancer, a high expression level of *STIM1* was reported and knockdown of *STIM1* and/or inhibition of SOCE inhibited cell proliferation and increased cisplatin-induced apoptosis (Ge et al., 2019; Li et al., 2013).

In pancreatic adenocarcinoma cell lines, *STIM1* was found to play an anti-apoptotic role where knockdown of *STIM1* showed an increase in cell death induced by chemotherapy such as 5-fluorouracil (5-FU) or gemcitabine (Kondratska et al., 2014). Regarding the mechanisms through which *STIM1* and SOCE could enhance cancer cell proliferation and survival, the *Akt*-mediated *STIM1* impact on cell proliferation and survival was reported in hepatocellular carcinoma cells (Zhao et al., 2020). Furthermore, Feldman et al. (2010) reported that *STIM1* knockdown in malignant melanoma cells produced Akt inactivation and increased susceptibility of cells to apoptosis. Another report on a human melanoma by Umemura et al. (2014) explained the effect of *STIM1* and SOCE in promoting cell proliferation through enhancing the CaMKII/Raf-1/ERK signaling pathway.

2.3.2 Calcium and ROS crosstalk in cancer

Accumulative evidence established the calcium and ROS interplay and reported that calcium signaling stimulates cellular ROS production and oxidants regulate calcium-related proteins and channels such as STIM1, IP_3R, and transient receptor potential (TRP) channels (Dejos et al., 2020; Hempel & Trebak, 2017). The effect of calcium on mitochondrial ROS (mtROS) production is dependent on mitochondrial metabolic state whereas mitochondrial Ca^{2+} overload enhances mtROS production independently on the metabolic state of mitochondria (Görlach et al., 2015; Li et al., 2013). Further evidence suggests that ROS production through NADPH oxidases also influences by Ca^{2+} ion which could be directly through calcium-binding domains as in the NOX5, DUOX1, and DUOX2 or indirectly through cytosolic activator, PKCβ1 and S100A8/A9 proteins, as in NOX1 and NOX2 (Görlach et al., 2015, Bréchard et al., 2013).

In non-cancer cells, oxidative stress initiates cytoplasmic and mitochondrial Ca^{2+} overload through numerous mechanisms, as the following: 1) Activate IP_3R to enhance Ca^{2+} release from ER and shuttle to mitochondria, 2) Bind to redox-sensitive *STIM1* cysteines (C49, C56) and stimulate Ca^{2+} influx, 3) Stimulate TRP channels on the plasma membrane to enhance Ca^{2+} influx, 4) Inhibit extrusion of cytoplasmic Ca^{2+} through plasma membrane Ca^{2+} ATPase. All these ROS-induced events lead to cytoplasmic and mitochondrial Ca^{2+} overload, which initiates apoptotic cascades through enhanced mitochondrial swelling and cytoplasmic c release. Cancer cells have the ability to adapt to oxidative stress and maintain sub-lethal levels of ROS. Furthermore, this type of cellular adaptation of cancer cells was reported to be linked to ROS/Ca^{2+} interaction (Hempel & Trebak, 2017).

In breast cancer, reducing mitochondrial calcium uptake through deletion of the mitochondrial Ca^{2+} uniporter (MCU) in MDA-MB-231 xenografts reduced mitochondrial ROS production and inhibited tumor growth and metastasis (Feno et al., 2019). Transient receptor potential A1 (TRPA1), a ROS-sensitive calcium channel, adapts cancer cells to therapy-induced oxidative stress through up-regulation of calcium-dependent pro-survival signaling proteins (RAS/ERK, PI3K/AKT) and increases the expression of the anti-apoptotic protein MCL-1 (Takahashi et al., 2018). Bcl-2 binding to IP3R slows IP3-induced Ca^{2+} outflow from the ER, which reduces mitochondrial Ca^{2+} concentrations and inhibits cancer cell apoptosis (Marchi & Pinton, 2016). In hepatocarcinoma cell lines, suppression of Hypoxia-inducible factor 1-alpha suppressed xenograft hepatic tumorigenesis through reduced *STIM1* levels (Bhardwaj et al., 2016).

2.3.3 *STIM1* and SOCE in hematological malignancies

Despite well-established research on the significance of *STM1* and SOCE in the pathogenesis of a variety of solid cancers, studies on the role of *STM1* and SOCE in hematological malignancies are still limited. In multiple myeloma (MM), high *STIM1* and *Orai1* expression were found in MM cell lines, and suppression of *STIM1* and *Orai1* reduced cell growth and arrested the cell cycle (Wang et al., 2018). Another study on T cell acute lymphoblastic leukemia (T-ALL) found that deleting *STIM1* and *STIM2* in mice with T-ALL prolonged mice's survival through reducing the necroinflammatory response in organs infiltrated with leukemia and down-regulating pro-inflammatory pathways in leukemic T lymphoblasts (Saint Fleur-Lominy et al., 2018). In the myeloid tumor, *Orai1* and *Orai2* were found to be linked to HL-60 cell proliferation and FAK-mediated cell migration (Diez-Bello et al., 2017).

In another study, Orai3 was discovered to contribute to Tipifarnib-induced cell death in myeloid leukemia cells where it was highly expressed in Tipifarnib-sensitive compared to Tipifarnib-insensitive leukemia cells (Vashisht et al., 2015). A previous study performed on Non-Hodgkin B lymphoma cells revealed the key function of *STIM1* and *Orai1* in the invasion and migration of diffuse large B cell lymphoma (DLBCL) cells (Latour et al., 2017). In chronic myeloid leukemia, *Orai1* expression was found to be higher than *STIM1* in Bcr-Abl expressing 32d cells, which could explain the decreased SOCE activity through lowering *STIM1*/*Orai1* binding stoichiometry (Cabanas et al., 2018). Therefore, all these data point the critical involvement of *STIM1* and SOCE in a variety of hematological malignancies, as well as the importance of studying *STIM1's* effect on AML cells.

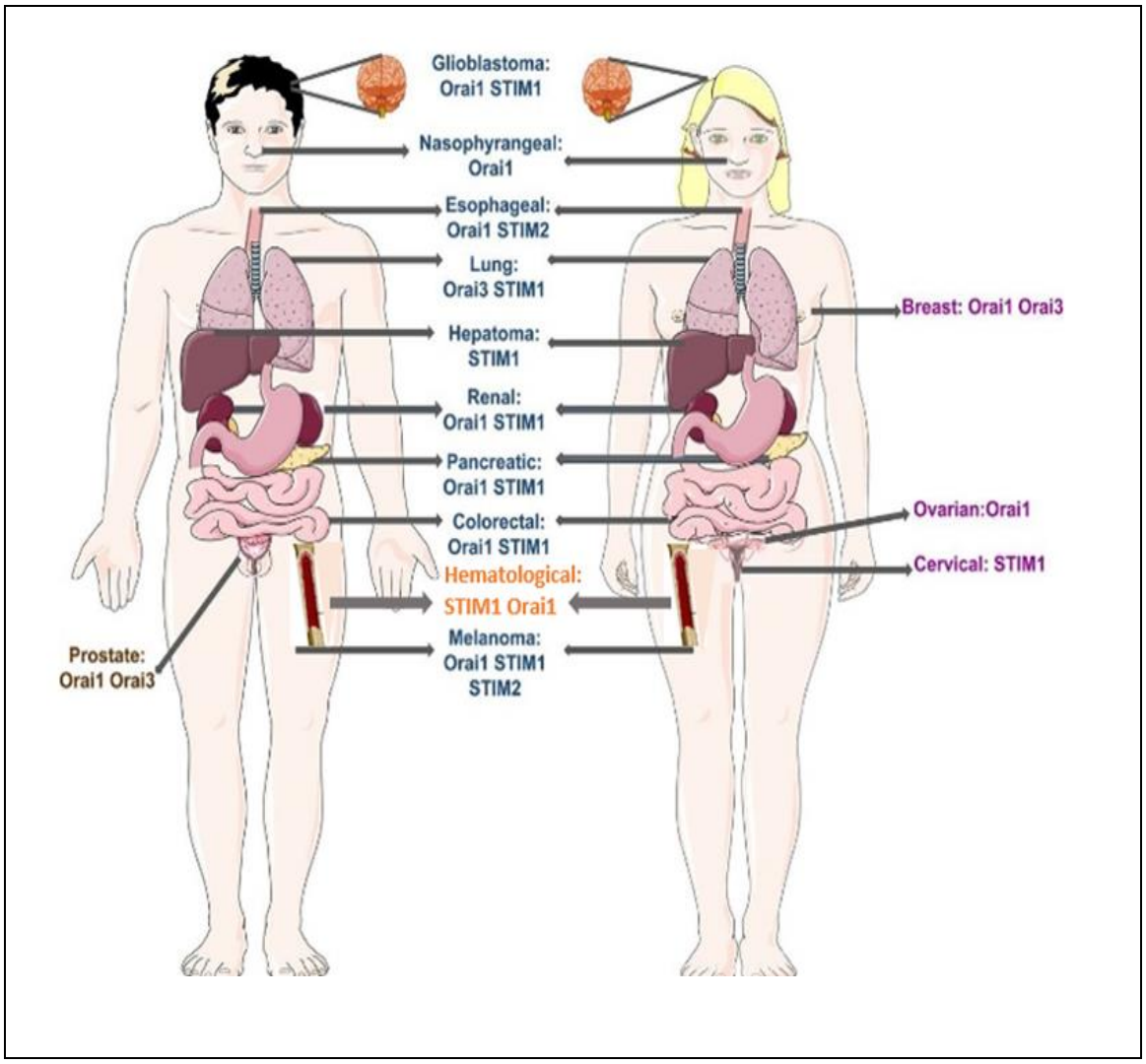

Figure 2.5 **The involvement of SOCE proteins, STIM and Orai, in different types of cancers**. STIM proteins (STIM1 and STIM2) and Orai proteins (Orai1-3) are involved in the tumerogenesis of many cancers that originate in various human tissues. (Adapted from Vashisht et al. 2015).

CHAPTER 3

METHODOLOGY

3.0 Introduction

This chapter represents the materials and methods used to perform this study. The chapter includes eight sections: the first section presents the general study materials, and the second section shows the experimental flow of the study. The third section describes human cell models, while the fourth section represents gene silencing work. The fifth-seventh sections explain the materials and methods used to assess the impact of *STIM1* silencing on the molecular, biochemical, and functional profiles of the cells, respectively. The last section provides the statistical methods used to analyze the results.

3.1 Sterilization of materials and solutions

All plasticwares including pipette tips and microcentrifuge tubes, were autoclaved at 121 ºC and pressure of 15 psi for 30 minutes prior to use. The electroporation cuvettes were sterilized with 70% (v/v) ethanol, then rinsed with deionized water, dried, and UV for 4-5 hours. The solutions, such as deionized water and phosphate buffer saline (PBS), were also sterilized through autoclaving at 121 ºC and pressure of 15 psi for 45 minutes before the experiment. The media, such as Roswell Park Memorial Institute Medium (RPMI-1640) (Sigma-Aldrich, USA), were prepared using autoclaved deionized water and filtered at the end of preparation using a filter system, vacuum filter 0.22 µm.

3.2 Experimental workflow

The experimental workflow of present study is shown in Figure 3.1.

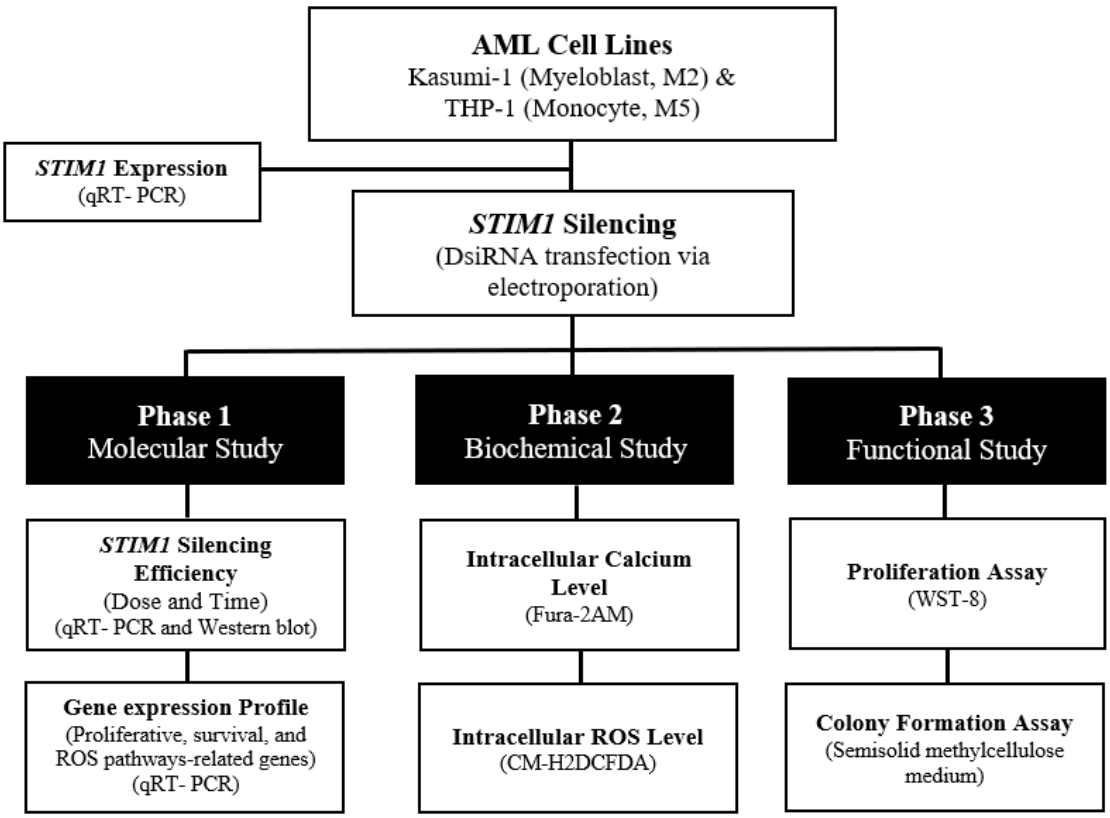

Figure 3.2 Methodology workflow of the present study.