

PROFILING OF GENE AND EXOSOMAL MICRORNA AS THERAPY TARGETS AND BIOMARKERS OF CISPLATIN RESISTANCE IN TRIPLE NEGATIVE BREAST CANCER STEM CELL-LIKE SUBPOPULATION

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Abstract

Breast cancer is the leading cause of death in women worldwide. Among different breast cancer subtypes, triple negative breast cancer (TNBC) which is defined by lack of oestrogen receptor, progesterone receptor and human epidermal growth factor receptors 2 possesses more aggressive behaviour than other breast cancer subtypes. Besides, TNBC is strongly associated with recurrence of metastatic breast cancer. One of the effective drugs used for TNBC treatment is cisplatin that inhibits DNA replication and dissociates p63 and p73, the transcription factors that are co-expressed exclusively in TNBC, thus leading to apoptosis of TNBC. However, the efficacy of cisplatin is compromised over time, resulted in cisplatin resistance and eventually cancer relapse. Cancer stem cells (CSCs) was proposed to contribute to the development of cisplatin resistance in breast cancer.

A short non-coding RNA namely microRNA, is now recognised of being capable to interfere with the onset and progression of cancer tumour via epigenetic regulation of gene expression. Furthermore, microRNAs can be packaged into exosome, forming exosomal-miRNAs that can circulate in various body fluid. As such, screening of the dysregulated profile of highly stable exosomal-miRNAs shows potential role in breast cancer diagnosis, prognosis and therapeutic as the acquirement of exosomal-miRNAs is non-invasive. Therefore, this study aims to investigate the role of CSC-like cells in cisplatin resistance, followed by elucidation of the exosomal-miRNAs profile of cisplatin-treated CSC-like cells for the discovery of potential biomarkers to monitor cisplatin resistance in breast cancer.

TNBC cell line, MDA-MB-231 was used in this study. CSC-like cells were first isolated based on expression of breast CSCs surface markers ($CD24^{-}/CD44^{+}$) from parental cells and the therapeutic effect of cisplatin on CSC-like cells was compared to that of the parental cells first via phenotypic characterisation. Next, dysregulation of CSC-correlated genes in treated CSC-like cells was

identified via PCR array. Followed by that, two platforms which were next generation sequencing (NGS) and Nanostring were used to decipher the exosomal-microRNAs profile in treated CSC-like cells.

Under the same cisplatin treatment condition (56μM of cisplatin for 48 hours), treated CSC-like cells retained higher expression of breast CSCs markers ($CD24^{-}/CD44^{+}$ and $ALDH1A1^{+}$), displayed lower G2/M phase cell arrest, sub G0/G1 event and late apoptosis/necrosis as compared to treated parental cells. In comparison to treated parental cells, treated CSC-like cells also demonstrated higher self-renewability. In correlation to its phenotypic characteristics, dysregulation of JAK2, SMO, PTCH1, GSK3β, NOTCH2, DLL1 and WNT1 detected in treated CSC-like cells were associated with JAK/STAT, Hh and angiogenesis signalling pathways and were suggested to contribute to higher drug resistance and cell stemness in treated CSC-like cells as compared to treated parental cells. Not only that, cisplatin treatment induced upregulation of exomiR-196a-5p, exomiR-939-5p and downregulation of exomiR-206 solely in treated CSC-like cells, which may attenuate the cytotoxicity of cisplatin, reflected by lower apoptotic event and higher self-renewability in CSC-like subpopulation in MDA via modulation of NOTCH2, GSK3β and TWIST1. Together, these data showed that CSC-like cells were more tolerant to the cytotoxic effect of cisplatin as compared to parental cells which could be modulated by dysregulated CSC-correlated genes and exosomal-miRNAs in treated CSC-like cells.

In addition, exo-miR-30a-5p, miR-28-3p, miR-126-3p and miR-941 were significantly dysregulated in CSC-like subpopulation detected by both NGS and Nanostring. Therefore, this subset of microRNAs could serve as potential candidates for development of prognostic biomarkers and therapeutic targets of cisplatin-resistant TNBC.

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DECLARATION

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ABBREVIATIONS

AJCC	American Joint Committee on Cancer
3'-UTR	3’-untranslated region
5FU	5-fluouracil
ABC	ATP-binding cassette
ABCG2	ATP-binding cassette subfamily G member 2
ABCB5	ATP Binding Cassette Subfamily B Member 5
ACTB	beta-actin
ADAMs	A disintegrin and metalloproteinases
Ago	Argonaute
AKT	Protein kinase B
AKT3	AKT Serine/Threonine Kinase 3
ALDH1	Aldehyde dehydrogenase 1
AML	acute myeloid leukemia
AP-1	Activator protein 1
APAF1	apoptotic protease activating factor 1
APC	Adenomatous polyposis coli
ASPP2	apoptosis-stimulating of p53 protein 2
ATF-2	Activating transcription factor 2
ATP	Adenosine triphosphate
ATR	Ataxia telangiectasia and Rad-3- related protein
ATRA	all-trans-retinoic acid
B2M	beta-2-microglobulin
BAX	B-cell lymphoma 2 Associated X
BCL11A	B-cell lymphoma/leukemia 11A
BCL-2	B-cell lymphoma 2
BCL2L1	BCL2-like 1
BCRP	Breast cancer resistance protein
BRCA	Breast cancer gene
CaMKII	Calcineurin and calcium/calmodulin-dependent protein kinase II
CD	Clusters of differentiation
CDH1	adenomatous polyposis coli/C activator protein
CE	Capillary electrophoresis
cGMP	cyclic guanosine monophosphate
CHMP4C	Charged multivesicular body protein 4c
CK1	Casein kinase 1α/β
CLL	Chronic lymphocytic leukemias
CRC	colorectal cancer
CSCs	Cancer stem cells
CSL/RBPJ	CBF1, Suppressor of Hairless, LAG-1/recombination signal binding protein for immunoglobulin k J region
CTR1	Copper transporter receptor 1
CXCR4	C-X-C chemokine receptor type 4
DAAM1	Dvl-associated activator of morphogenesis 1
DCIS	Ductal carcinoma *in situ*
DEAB	diethylaminobenzaldehyde
DHH	Desert Hh
DLL	Delta-like ligand
DNA	Deoxyribonucleic acid
DNMT3A	DNA methyltransferase 3A

Drosha-DGCR8	Drosha-DiGeorge syndrome critical region gene 8
DSF	disulfiram
DUSP6	dual-specific phosphatase 6
Dvl	Dishevelled
EGF	Epidermal growth factor
EGFR	Epidermal growth factor receptor
ELV	exosome like vesicle
EMT	Epithelial–mesenchymal transition
ER	Oestrogen receptor
ErbB	erythroblastic leukemia viral oncogene homologue
ERKs	Extracellular signal-related kinases
ERα	Oestrogen receptor alpha
ESCRT	Endosomal sorting complex required for transport
ETV1	ETS variant 1
EV	Extracellular vesicles
ExomiRNAs	Exosomal-miRNAs
EZH2	Enhancer of Zeste Homolog 2
FACS	Fluorescence Activated Cell Sorting
FADH2	Flavin adenine dinucleotide
FAO	fatty acid β-oxidation
FasL	Fas ligand
FC	Fold Change
FFPE	Formalin-fixed paraffin-embedded
FGF	Fibroblast growth factor
FOXO1	Forkhead box protein O1
FZD	Frizzled
GAPDH	Glyceraldehyde 3-phosphate dehydrogenase
GDV	gene delivery vector
GFP	Green fluorescent protein
GLIA	Active form of GLI
GliFL	full-length Gli
GliR	Gli repressor
GO	gene ontology
GSH	Glutathione
GSK3	Glycogen synthase kinase 3α/β
GSK3β	Glycogen synthase kinase 3 beta
HA	Hyaluronic acid
Hh	Hedgehog
HIF1A	Hypoxia Inducible Factor 1 Subunit Alpha
HKG	Housekeeping genes
HME	human mammary epithelial
hnRNP	heterogeneous nuclear ribonucleoprotein
HOXD10	Homeobox D10
HPRT1	hypoxanthine phosphoribosyltransferase 1
HR	homologous recombination
HSP9	Heat shock proteins 9
HSPs	Heat shock proteins
IC_{50}	half maximal inhibitory concentration
ICAM-1	Intercellular adhesion molecule-1
IHH	Indian Hh
IL	Interleukin

ILVs	Intraluminal vesicles
ITGA6	Integrin subunit alpha 6
JAG	Jagged ligand
JAK/STAT	Janus kinase/ Signal transducer and activator of transcription
JNKs	c-Jun N-terminal kinases
KDM6B	lysine (K)-specific demethylase 6B
KEGG	Kyoto Encyclopedia of Genes and Genomes
Ki67	Nuclear non-histone protein
KITLG	KIT ligand
KLF17	Krueppel-like factor 17
KLF4	Kruppel-like factor 4
KRAS	Ki-ras2 Kirsten rat sarcoma viral oncogene homolog
LAMP1/2	Lysosomal-associated membrane protein 1/2
LBPA	Lysobisphosphatidic acid
LDHA	Lactate dehydrogenase A
LepRb	Leptin activated long form of leptin receptor
LIN28B	Lin-28 Homolog B
LNA	locked nucleic acid
LRP	Low-density lipoprotein-related protein
LZTFL1	Leucine Zipper Transcription Factor Like 1
MAPK	Mitogen-activated protein kinases
MCL-1	Myeloid cell leukemia 1
MDA	MDA-MB-231
MDR1	Multidrug resistance protein
MDSCs	Myeloid-derived suppressor cells
MHC	Major histocompatibility complex
miRISC	miRNA-containing RNA-induced silencing complex
miRNAs	MicroRNAs
MMP-1	Matrix metalloproteinase-1
mRNA	Messenger RNA
MRP2	multi drug resistance-related protein 2
MSCs-Exo	mesenchymal stem cells-exosome
MTMR3	myotubularin-related protein 3
mTOR	mammalian target of rapamycin
MTT	3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyl tetrazolium bromide
MUC1-C	Mucin 1 transmembrane C-terminal
MVB	Multivesicular body
NADH	Nicotinamide adenine dinucleotide
NCID	Notch intracellular domain
NGS	Next-generation sequencing
NLK	nemo-like kinase
nMase-2	Neutral sphingomyelinase-2
NOV	nephroblastoma overexpressed
NQO1	NAD (P) H-quinone oxidoreductase 1
NRF2	nuclear factor erythroid 2-related factor
NSCLC	non-small-cell lung cancer
NTA	Nanoparticle tracking analysis
OCT4	Octamer-binding transcription factor 4
OD	Optical density
ONECUT2	One Cut Homeobox 2
One-way ANOVA	one-way analysis of variance

P27	Cyclin-Dependent Kinase Inhibitor 1B
PAGE	polyacrylamide
pCR	pathological complete response
PDX	Patient derived xenografts
pgk1	Phosphoglycerate kinase 1
PGRL	p120 catenin, radixin, and immunoglobulin superfamily member 8
piRNA	piwi-interacting RNAs
PKC	Protein kinase C
PLAT	Plasminogen Activator, Tissue Type
PLC	Phospholipase C
pMDA	Parental MDA-MB-231
pol II/III	Polymerase II/III
PR	Progesterone receptor
pre-miRNAs	Precursor transcripts of miRNAs
pri-miRNAs	Primary transcripts of miRNAs
PS	Phosphatidylserine
pSTAT3	phospho-STAT3
PTCH	Patched
PTEN	Prime Time Entertainment Network
PTPRC	receptor-type protein tyrosine phosphatase
QC	quality control
qPCR	Quantitative polymerase chain reactions
Rabs	Ras-like small GTPase family
RGS3	regulator of G-protein signalling 3
RISC	RNA-induced silencing complex
RNA	ribonucleic acid
ROCK	Rho-associated kinase
ROS	Reactive oxygen species
RPL10	ribosomal protein L10
RPLP0	ribosomal protein lateral stalk subunit P0
RT-qPCR	Reverse transcriptase-real time polymerase chain reaction
S100A4	S100 calcium-binding protein A4
SD	Standard deviation
SEER	Surveillance, Epidemiology, and End Results
SEM	standard error of mean
SFE	Sphere forming efficiency
sFRP4	secreted frizzled-related protein 4
SHH	Sonic Hh
siRNA	Small interfering RNAs
SMAD2	SMAD family member 2
SMO	Smoothened
SOCS3	suppressor of cytokine signalling 3
SOX2	SRY (sex determining region Y)-box 2
STAM1	Signalling transducing adaptor molecule 1
STAT3-CPT1B-FAO	STAT3-carnitine palmitoyltransferase 1B-fatty acid β-oxidation
STAT5B	Signal Transducer And Activator Of Transcription 5B
stRNA	Small temporal RNA
SUFU-GLI	Suppressor of fused-glioma-associated oncogene homolog
TAMs	Tumour-associated macrophages
TaqMan-qPCR	TaqMan-real time polymerase chain reaction

TCF/LEF	T-cell factor/lymphoid enhancer factor
TDEs	Tumour-derived exosomes
TEM	Transmission electronic microscopy
TFs	Transcription factors
TGFBR2	transforming growth factor, beta receptor II
TNBC	Triple negative breast cancer
TRBP	Transactivation response RNA binding protein
Tsg101	Tumour susceptibility gene 101
TSPAN8	tetraspanin-8
TUG1	taurine upregulated gene
TWIST1	Twist-related protein 1
uPA	urokinase-type plasminogen activator
UTR	Untranslated region
VPS4B	Vacuolar protein sorting-associated protein 4B
VTA1	Vacuolar protein sorting-associated protein
ZEB1	Zinc Finger E-Box Binding Homeobox 1
β-TrCP	β-Transducin Repeat-Containing Protein

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Chapter 1

Introduction

Triple negative breast cancer (TNBC) is a breast cancer subtype defined by their lack of oestrogen receptor and progesterone receptor, and the absence of amplification of human epidermal growth factor receptors 2 (Liao et al., 2018). Globally, TNBC accounts for approximately 10-24% of all breast cancer cases, and is more common in Asian countries (Krishnamurthy et al., 2012). In Malaysia, the incidence of TNBC has been reported to range between 12.3% to 17.6% of the total breast cancer cases (Abdul Aziz et al., 2020; Kanapathy Pillai et al., 2012; Tan et al., 2009). TNBC displayed a more aggressive behaviour than other breast cancer subtypes, strongly associated with recurrence of metastatic breast cancer, where metastasis is a major cause of patient morbidity and mortality (André & Zielinski, 2012; Seyfried & Huysentruyt, 2013).

Due to the absence of all 3 receptors, treatment for TNBC patients is highly challenging as no defined targets are available. One of the strategies used to tackle TNBC is to target its DNA repair complex via platinum agents, such as cisplatin that inhibits DNA replication of TNBC (Wahba & El-Hadaad, 2015). Not only that, cisplatin is able to dissociate p63 and p73, the transcription factors that are co-expressed exclusively in TNBC. The binding of these proteins suppressed pro-apoptotic activities in TNBC. Therefore, dissociation of the proteins would activate p73-dependent transcription of pro-apoptotic Bcl-2 family members, leading to apoptosis (Leong et al., 2007). Supporting this treatment strategy for TNBC, treatment regime consisting cisplatin is showing promising results to most of the TNBC patients (Amos, Adamo, & Anders, 2012; Gluz et al., 2009; Leong, Vidnovic, DeYoung, Sgroi, & Ellisen, 2007).

Despite good response from patients for cisplatin in the beginning of treatment, development of cisplatin resistance often occurs over time and resulted in cancer relapse (Basu & Krishnamurthy, 2010; Isakoff, 2010; Oakman et al., 2010). Up to date, development of cisplatin resistance remained unresolved, resulting as a major obstacle for effective clinical application

using cisplatin (Zheng et al., 2017). In fact, recurred cancer usually become more aggressive and highly resistant to therapeutics, which greatly reduce the lifespan of TNBC patients (Byrski et al., 2014; Turner et al., 2004).

On the other hand, many studies have demonstrated the presence of cancer stem cells (CSCs) as one of the leading cause for drug resistance in cancer patients. In correlation to breast cancer, a study conducted by Shafee et al. (2008) demonstrated CSCs as the contributor for cisplatin resistance developed in basal-like breast cancer. With that, presence of CSC in TNBC could be one of the factors for the development of cisplatin resistance. Apart from displaying higher chemoresistance, CSCs are featured as highly metastatic and self-renewable. For example, previous studies demonstrated overexpression of apoptosis-related proteins in CSCs including survivin (Carter et al., 2012; Jin et al., 2008), cellular inhibitors of apoptosis, inhibitors of apoptosis protein (IAP) (Liu et al., 2006) and cell cycle regulatory components that inhibits apoptosis, leading to higher chemoresistance (Abdullah & Chow, 2013). Besides, studies have shown that CSCs are more likely to metastasise to secondary site, later generate a secondary tumour via its self-renewability (Velasco-Velazquez, Popov, Lisanti, & Pestell, 2011).

Over the past decades, researchers have demonstrated that high CD44 and low CD24 expression ($CD24^{-}/CD44^{+}$) is the standard biomarker for breast CSCs. These biomarkers were first discovered by Al-Hajj et al. (2003), showing as low as 100 cells expressing $CD24^{-}/CD44^{+}$ were able to generate tumour in mice while thousands of non $CD24^{-}/CD44^{+}$ cells failed to do so. Followed by his discovery, many have utilised this CSCs phenotype as the biomarkers for breast CSCs isolation (Abraham et al., 2005; Liu et al., 2010; Zhao et al., 2016). Indeed, Ghebeh et al. (2013) compared the stem-cell properties of CSCs isolated from different biomarkers and concluded that $CD24^{-}/CD44^{+}$ phenotype as the predominant biomarkers for breast CSCs among other biomarkers.

In addition to that, it is now recognised that microRNAs can interfere with the onset and progression of cancer tumour via epigenetic regulation of gene expression. For instance, a set of microRNAs were significantly dysregulated in MCF-7 cells resistant to cisplatin. These

differentially expressed miRNAs are involved in the control of cell signalling, cell survival, DNA methylation and invasiveness (Pogribny et al., 2010). In regards to CSCs, accumulated studies demonstrated distinctly regulated microRNAs including miR-200 cluster, miR-183 cluster, let-7, miR-600, miR-30, miR-221-222 cluster, miR-142, miR-214 and miR-31 are required for regulation of proliferation, tumour initiation, differentiation, cell stemness, apoptosis, metastasis and to govern fate decision in breast CSCs (El Helou et al., 2017; Lv et al., 2017; Shimono et al., 2015; Yu et al., 2010)

Not only that, microRNAs are found actively sorted and transported in exosome, a nano-sized (10-200 nm) endosome-derived vesicle secreted by cell into extracellular space for intercellular communication (Doyle & Wang, 2019; Val et al., 2017; Valadi et al., 2007). Exosomes are found to be present in abundance in different types of cancer patient and in cancer cell supernatants (Hessvik et al., 2012; Pigati et al., 2010). In fact, a plethora of evidence indicated that exosome-mediated cell-cell communication can promote tumour initiation, metastasis, and therapy-resistance in cancer cells within the tumour microenvironment (Azmi et al., 2013; H.-G. Zhang & Grizzle, 2014). For example, exosomal-miRNA secreted by drug-resistant cancer cells had been shown to enhance drug tolerance of non-resistant cancer cells (Chen et al., 2014; Mao et al., 2016; Shen et al., 2017; Wei et al., 2014; Yu et al., 2016; Zhong et al., 2016) while exosomal-microRNAs secreted by metastatic breast cancer cells resulted in induction of metastatic ability in non-metastatic tumour cells (Le et al., 2014; Singh et al., 2014). Although the research on exosome is still in its infancy, these new findings collectively suggest that exosomes may be a medium of communication between native and CSCs that contribute to resistance to cytotoxic stimuli or even cancer relapse. More importantly, exosomal-microRNAs that are readily present in body fluids can serve importantly for cancer diagnosis, prognosis, and therapeutics (Taylor & Gercel-Taylor, 2008; Vlassov et al., 2012). Prognostic biomarkers are crucial for identification of TNBC patients at higher risk of experiencing drug resistance, relapse or metastasis for better strategize on the surveillance and management of the patients (Abdul Aziz et al., 2020).

Therefore, this study aims to find out the contribution of CSCs subpopulation to cisplatin resistance in TNBC and to profile exosomal-miRNAs that are dysregulated in CSCs as compared to parental cells in response to cisplatin treatment. These exosomal-microRNAs that are differentially regulated may serve as important biomarkers for prognosis of cisplatin resistance. Likewise, signalling pathways that are associated with these microRNAs can also be identified to support more effective treatment strategies.

To address the research questions, CSC-like cells isolated from TNBC cancer cell line, MDA-MB-231 was used in this study to investigate cisplatin-induced alteration of phenotypic and genotypic characteristics, followed by transcriptomic profiling of differentially regulated exosomal-miRNAs to unveil the association of CSC-like cells in MDA-MB-231 to the response to cisplatin treatment.

Core objectives:

1) To investigate the effect of cisplatin treatment on phenotypic and genotypic characteristics of CSC-like cell as compared to parental MDA-MB-231 breast cancer cell. The specific objectives to achieve this core objective are as stated below:

i. To identify the phenotypic characteristics of isolated CSC-like subpopulation

ii. To assess the cytotoxic effect of cisplatin on CSC-like subpopulation

iii. To study the dysregulation of mRNAs expression in CSC-like subpopulation induced by cisplatin treatment

2) To elucidate exosomal-microRNAs secreted by CSC-like cells in response to cisplatin treatment in MDA-MB-231 breast cancer cell. Specific objectives to fulfil this core objective are as stated below:

i. To identify dysregulation of exosomal-microRNA in CSC-like subpopulation induced by cisplatin treatment

ii. To predict the corresponding signalling pathways correlated to the differentially expressed exosomal-microRNA in CSC-like subpopulation induced by cisplatin treatment

iii. To identify potential prognostic biomarkers and therapeutic target for cisplatin resistance in association with CSC-like subpopulation

Chapter 2

Literature review

2.1 Breast cancer

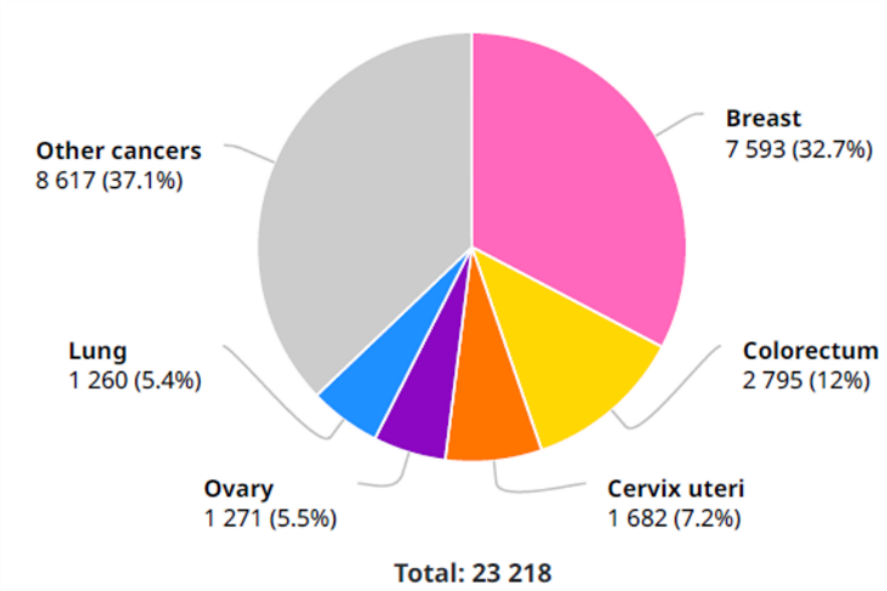
2.1.1 Incidence of breast cancer

Breast cancer is a disease induced by abnormal growth of cells which divided uncontrollably in breast tissue (Cooper GM, 2000). The onset of cancer is determined by both internal factors including genetic defects or inheritable mutations and environmental factors such as lifestyle, diet and exposure to radiation or carcinogenic substances (Anand et al., 2008). Breast cancer often occurs in women but does exist in male with very rare incidence of less than 1% (Ly et al., 2013).

Being the most common female cancer with high mortality rate, breast cancer is impacting 2.1 million women each year (24.2%) of all cancers among women. In 2018, breast cancer was estimated to cause approximately 15% of all cancer deaths in women. While breast cancer rates are higher among women in more developed regions, rates are increasing in nearly every region globally (Bray et al., 2018).

In Malaysia, breast cancer comprised 32.7% of all cancers in women of all ages followed by a drastic reduced rate to 12% of colorectum cancer (**Figure 2.1A**). Besides, breast cancer is the leading cause of death among Malaysian (**Figure 2.1B**). A summary on breast cancer study in Malaysia postulated the correlation of higher risk of breast cancer to consumption of oral contraceptives, not breastfeeding, family history and nulliparity of Malaysian women (Yip et al., 2014). There are a few extrinsic factors that could contribute to the onset of breast cancer such as exposure to radiation, lack of exercise and smoking (Anand et al., 2008).

A: Number of new cases in 2018, females, all ages



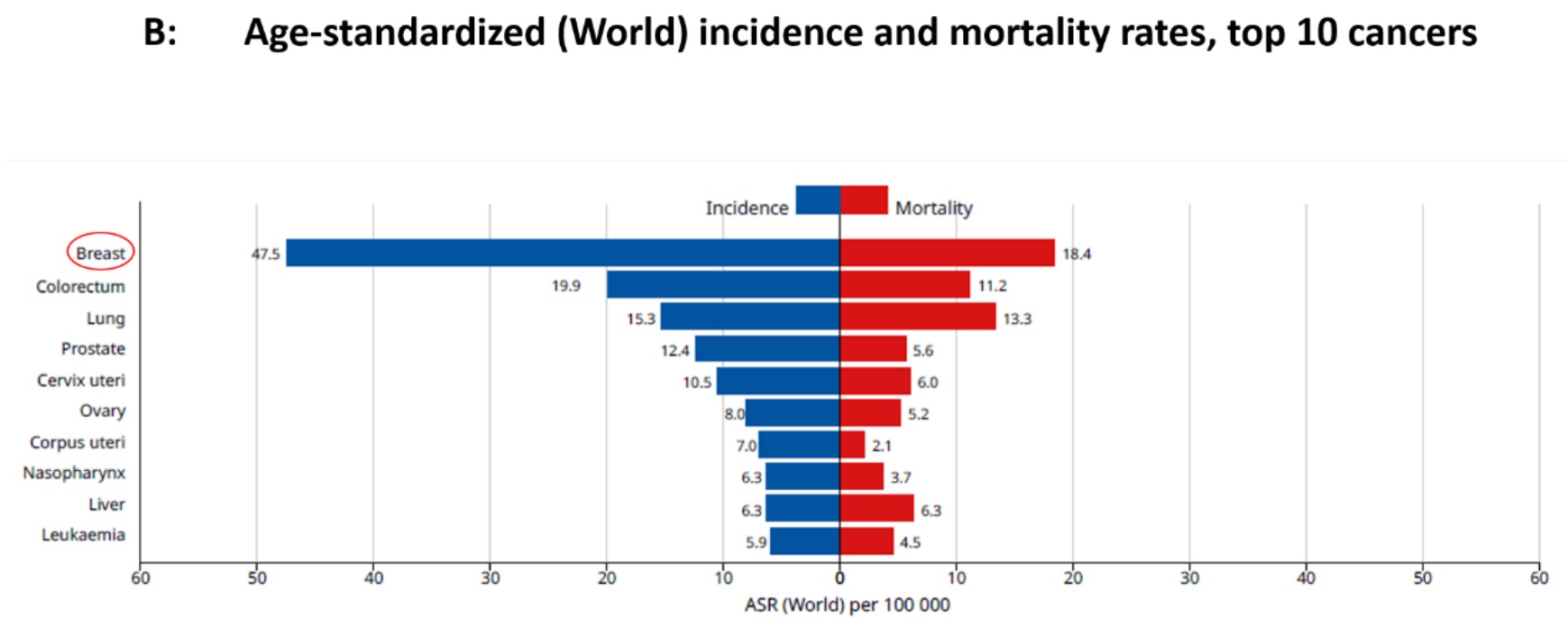


Figure 2.1: Incidence and mortality rate of breast cancer patients in Malaysia (2018). Adapted from, World Health Organization. (2018). Retrieved April 5, 2020, from https://gco.iarc.fr/today/data/factsheets/populations/458-malaysia-fact-sheets.pdf

Breast cancer is categorised into different stages based on the latest staging guidelines provided by American Joint Committee on Cancer (AJCC) which includes assessment on the size of tumour, presence of cancer in lymph nodes, metastasis, tumour grade, presence of hormonal receptors, expression of human epidermal growth factor receptor 2 (Her-2) and oncotype DX score (recurrence score) (Koh & Kim, 2019).

2.1.2 Different subtypes of breast cancer

There are four main subtypes of breast cancer including luminal A, luminal B, Her-2-enriched and triple negative breast cancer (TNBC) (Perou et al., 2000). Luminal subtype A is defined as oestrogen receptor (ER)- or progesterone receptor (PR)-positive, Her-2-negative and low expression of a nuclear non-histone protein (Ki67). This is the most common breast cancer subtype with the best prognosis (Badve et al., 2007). Next, luminal subtype B characterised with ER- or PR-positive status, either positive or negative regarding Her-2 and high expression of Ki67. Luminal subtype B is prominent in elderly patients with breast cancer, where ductal carcinoma is common (Tang et al., 2015). Her-2-positive breast cancers overexpress Her-2 but are negative for ER and PR (Fragomeni et al., 2018). TNBC is classified by lack of ER, PR and proto-oncogene Her-2, also more aggressive as compared to other subtypes. This is proven by previous study that shown higher incidence of distant metastasis for TNBC to lung and liver as compared to non-TNBC (André & Zielinski, 2012; Oakman et al., 2011; Podo et al., 2010). In Malaysia, TNBC accounts for approximately 17% of all reported breast cancer incidences (Dean & Rhodes, 2014). With high metastatic ability and lack of treatment targets, it is no surprise that this subtype of breast cancer is known to have poorer prognosis with limited treatment options (Chacón & Costanzo, 2010; Mehanna et al., 2019).

2.1.3 Triple negative breast cancer

Treatment for early stage TNBC involved breast-conserving surgery or a mastectomy if the size of tumour is small. Radiation followed by surgery will be given to patients with large tumour or detected cancer in lymph node. Besides, chemotherapy might be applied before surgery

(neoadjuvant chemotherapy) to shrink large tumour before surgery. If residual cancer cells are detected after surgery, patients are advised to take oral chemo drug or might need to proceed with chemotherapy after surgery (adjuvant chemotherapy) to minimise the chances of cancer relapse. Chemotherapy drugs such as anthracyclines, taxanes, capecitabine, gemcitabine and eribulin are often given to patients with late stage of TNBC. However, for TNBC patients with breast cancer gene (BRCA) mutation and those who no longer responds to common breast cancer chemo drugs, addition of platinum-based drug such as cisplatin to the regime is one of the treatment options (Isakoff, 2010; Koshy et al., 2010).

MDA-MB-231 is one of the most commonly utilised mammalian cell line for *in vitro* breast cancer studies (Han et al., 2019; Lee et al., 2014; Yan et al., 2018). MDA-MB-231 cell line was established from pleural effusion of a 51-year-old caucasian woman with metastatic mammary adenocarcinoma isolated at M D Anderson Cancer Center (Cailleau et al., 1978). MDA-MB-231 is highly aggressive, ER, PR, Her-2 and E-cadherin negative with mutated p53, thus representing a good model of TNBC (Chavez et al., 2010; Liu et al., 2003). This cell line is often used to model late-stage breast cancer.

2.2 Cisplatin

2.2.1 Discovery of cisplatin

Cisplatin was first invented in 1844 by an Italian chemist Michele Peyrone and was previously known as Peyrone's chloride (Kauffman et al., 2010). However, it was accidental discovered as a cancer treatment by Barnett Rosenberg, a biophysical chemist in 1960s (Chen et al., 2009). When platinum electrode was used in a bacterial experiment, Rosenberg observed inhibited division of bacteria but they grew up to 300 times than their normal size. Further study using sarcoma mouse model displayed anti-proliferative potential of cisplatin (Rosenberg & VanCamp, 1970), subsequently approved for clinical usage as anticancer drug in 1978

(Hambley, 2007; Higby et al., 1974). Now, cisplatin is used in chemotherapy for many types of cancer including metastatic breast cancer (Jamieson & Lippard, 1999; Ozkan et al., 2012).

2.2.2 Mechanism of cisplatin

Cisplatin is a water-soluble planar coordination complex which consists of a platinum atom surrounded by 2 ammonia groups and 2 chlorine atoms (**Figure 2.2**). Upon entering cancer cell, chloride ions dissociated from the complex leaving the reactive complex to react with water followed by disruption of deoxyribonucleic acid (DNA) strand (Basu & Krishnamurthy, 2010). Dissociated reaction complex from cisplatin targets N7 of guanine and the binding of cisplatin to DNA generated structurally different DNA adducts and this process is irreversible (Munchausen & Rahn, 1975).

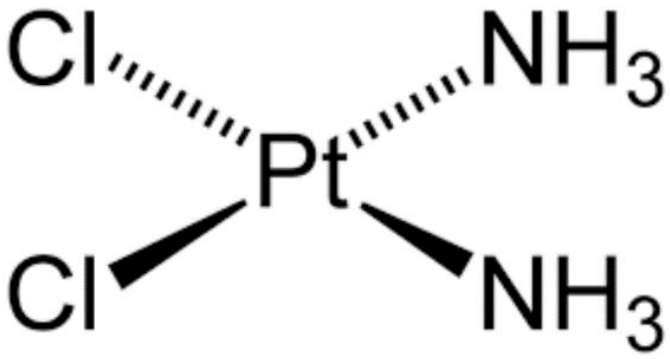


Figure 2.2: **Chemical structure of cisplatin**. Pt representing platinum atom, NH3 as ammonia group and Cl as chlorine atoms. Taken from Dai et al. (2017).

Cisplatin forms different DNA adducts via intra-strand crosslinking of two nucleobases of single DNA strand, inter-strand crosslinking of two different strands of one DNA molecule, chelate formation through N- and O-atoms of one guanine and DNA-protein crosslinks (Eastman, 1983). Other than inhibition of cell proliferation, cisplatin-induced DNA adducts can trigger a number of downstream signal transduction pathways involving p53, mitogen-activated protein kinases (MAPK) and p73 that contribute to cytotoxicity of cisplatin, which ultimately leading to apoptosis (Tanida et al., 2012) (**Figure 2.3)**.

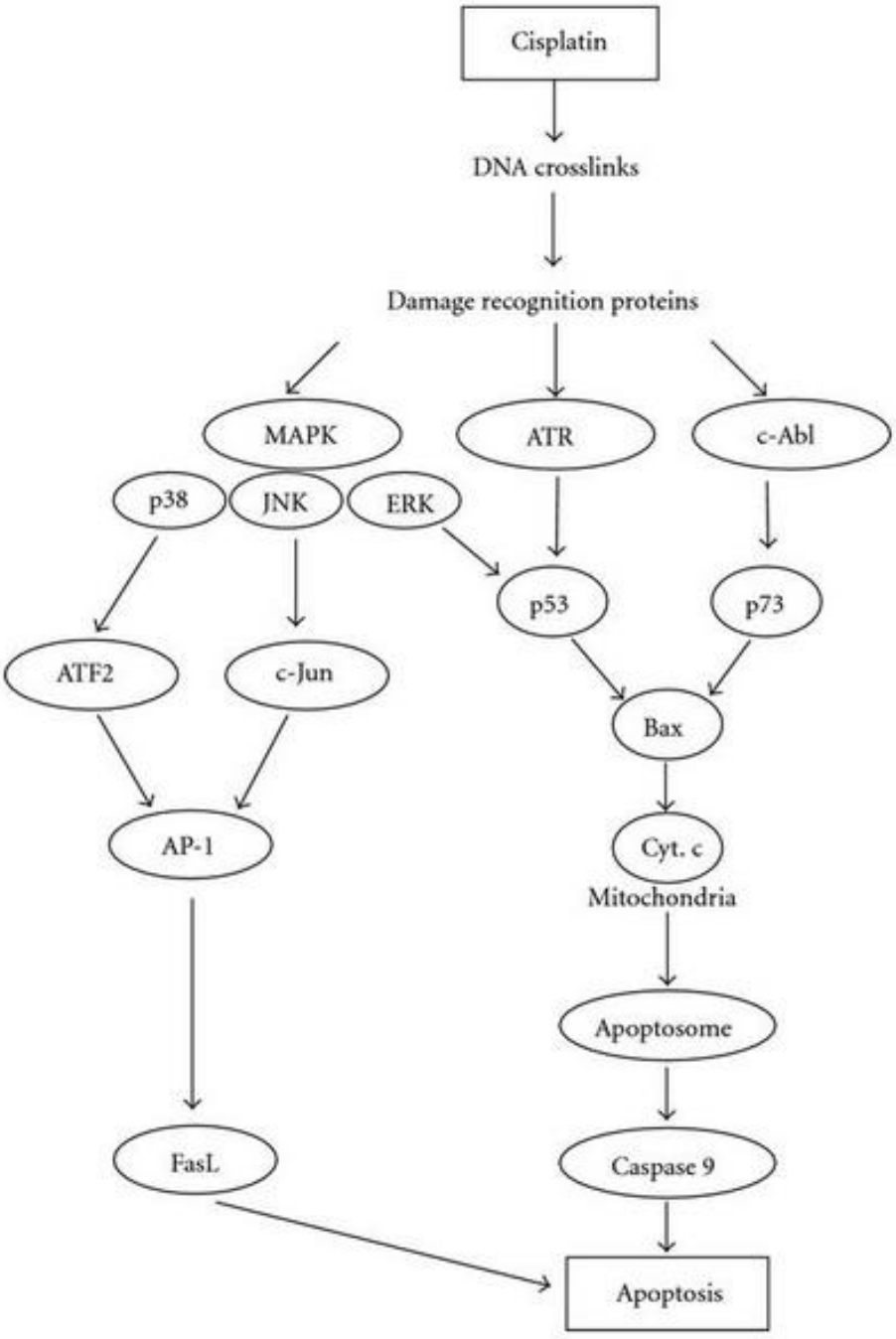


Figure 2.3: Signalling pathways contributing to cisplatin-induced apoptosis. Cisplatin-induced DNA damage can be recognised, that eventually triggers apoptosis through c-Abl- and p73-dependent cascade and/or two other pathways that involve MAPK and p53. Taken from Tanida et al. (2012).

The cisplatin-induced DNA damage is detected by damage recognition proteins results in DNA damage signal transmission to downstream signalling cascades. Transmitted signal subsequently induced other mechanisms including the activation of MAPK which in turn, trigger the activation of c-Jun N-terminal kinases (JNKs) and p38 kinases. The activated JNK/p38 kinases promote phosphorylation of transcription factors (TFs), c-Jun and activating

transcription factor 2 (ATF-2), which then bind to the activator protein 1 (AP-1) binding site in promoters of various genes. As the result, cisplatin is capable to induce apoptosis via Fas ligand (FasL) gene expression activated by AP-1 (Tanida et al., 2012). Other than that, activated MAPK will stimulate the activation of extracellular signal-related kinases (ERKs), results in phosphorylation of p53 TF at serine-15 (Persons et al., 2000). Similar to that, p53 can also be phosphorylated by ataxia telangiectasia and Rad-3- related protein (ATR) at serine-15 (Damia et al., 2001). Next, another TF, p73 plays a role as pro-apoptotic protein and is often found to be accumulated in cisplatin-treated cells (Tanida et al., 2012). p73 can be triggered by the activated oncogenic tyrosine kinase, c-Abl which is induced by cisplatin treatment (Yuan et al., 1999). Both the phosphorylated p53 and p73 will then activate the release of cytochrome c, which is promoted by Bax (Viktorsson et al., 2003). The release of cytochrome c triggers the formation of apoptosome assembly, leading to the activation of caspase 9, thus resulting in apoptosis (Sedletska et al., 2005) (**Figure 2.3**). In short, the efficacy of cisplatin is not merely contributed by impeded DNA replication associated with DNA-adduct, the cell responses triggered by cisplatin-induced DNA damage is another factor that induces apoptosis (Basu & Krishnamurthy, 2010).

Due to absence of treatment target in TNBC, cisplatin is applied to inhibit DNA replication, disrupt the DNA repair complex and induce apoptosis in TNBC (Wahba & El-Hadaad, 2015). Two TFs including p63 and p73 are co-expressed exclusively in TNBC where the binding of p63 to p73 suppresses pro-apoptotic activities. Cisplatin is able to counteract with p63-p73 associated anti-apoptotic mechanism by dissociating p63 from p73 and activates p73-dependent transcription of pro-apoptotic B-cell lymphoma 2 (Bcl-2) family members which leads to apoptosis in TNBC (Leong et al., 2007). Supporting this treatment strategy for TNBC, treatment regime consisting of cisplatin is showing promising results to most of the TNBC patients (Amos, Adamo, & Anders, 2012; Gluz et al., 2009; Leong, Vidnovic, DeYoung, Sgroi, & Ellisen, 2007).

2.2.3 Development of cisplatin resistance

Despite good response from patients for cisplatin in the beginning of treatment, development of cisplatin resistance often occurs over time resulting in cancer relapse (Basu & Krishnamurthy, 2010; Isakoff, 2010; Oakman et al., 2010). For example, patients who initially responded to cisplatin therapy often develop resistance due to activation of the homologous recombination (HR) DNA repair mechanism (Aloyz et al., 2002; Husain et al., 1998). In addition to the activation of DNA repair mechanism, cisplatin resistance studied in cultured cancer cells conferred resistance by disrupting cellular accumulation of cisplatin (Ishida et al., 2002), detoxification of cisplatin (Hill et al., 2019) and altering cell-cycle checkpoints (J. O. Lee et al., 2019; D.-W. Shen et al., 2012).

Reduced cellular accumulation of cisplatin compound is the result of reduced cisplatin uptake or retention in the cells. As the result, formation of DNA adducts is correspondingly decreased, reducing cytotoxicity and leads to higher resistance to cisplatin. Increased level of copper transporter receptor 1 (CTR1), which is a major influx transporter, was found mediating cisplatin uptake in yeast and mammals (Ishida et al., 2002). It has later been further confirmed in human cells that cisplatin triggered rapid degradation of CTR1 and diminished influx of cisplatin, results in resistance to cisplatin (Holzer et al., 2006; Lin et al., 2002). Furthermore, genetic knockout of CTR1 demonstrated cellular resistance to cisplatin *in vivo* (Howell et al., 2010). These studies showed that lower CTR1 expression led to lower platinum accumulation and, in most instances, more resistant to cisplatin.

Next, detoxification of cisplatin was found associated with glutathione (GSH), which hindered the cytotoxic effect of cisplatin in lung cancer (Lan et al., 2018; Silva et al., 2019). This GSH-associated protective mechanism against cisplatin could be due to its role as cofactor that facilitated multi drug resistance-related protein 2 (MRP2)-mediated cisplatin efflux in mammalian cells, demonstrated by MRP2-transfected cells that conferred cisplatin resistance (Cui et al., 1999; Ishikawa et al., 1994). Besides, GSH can bind to and inactivate cisplatin

through its highly reactive thiol group, preventing the drug from binding to DNA and induce damage (Rocha et al., 2016).

Cisplatin-induced DNA damage activated numerous pathways including activation of cell cycle checkpoints, which temporally induce a transient S phase arrest, followed by inhibition of the Cdc2-cyclin A or B kinase to affect a durable G2/M arrest (Shapiro & Harper, 1999; Shi et al., 1994). Cell cycle arrest is seen as inhibitory to the cytotoxic process, which is a conclusion that derives primarily from the increased cellular sensitivity to cisplatin upon abrogation of the G2/M checkpoint pharmacologically. Cell cycle arrest, as a generally accepted consequence of DNA damage, is necessary to enable the nucleotide excision repair (NER) complex to remove the adducts for cell survival (Siddik, 2003). Study found that testicular germ cancer cell was most sensitive to cisplatin in G2/M phase as all crucial events were restricted to this phase. For example, cisplatin-induced DNA-damage was sensed in G2/M phase, apoptotic process was initiated and eventually executed in this phase of cell cycle. On the other hand, the study suggested that inhibition of the cell cycle in G1/S phase, is likely to result in a protective effect against cisplatin, whereas cell arrest in G2/M may exert a synergistic effect (Mueller et al., 2006). These studies demonstrated that cell cycle arrest is beneficial for cell survival, whereby cell cycle arrest on earlier phase than G2/M phase could be a protective mechanism against cisplatin treatment.

Inefficient cisplatin treatment can lead to residual disease in patients, thereby worsen the overall survival rate especially for TNBC compared with non-TNBC (Liedtke et al., 2008).

2.3 Breast cancer stem cell

2.3.1 Discovery of cancer stem cell

Intriguingly, primary tumours only contribute minimally to cancer deaths whereas an estimation of 90% of mortality and morbidity in cancer is actually due to cancer metastasise to a distant site (Chaffer & Weinberg, 2011; Seyfried & Huysentruyt, 2013). In correlation to that, emerging evidences have suggested that a small subpopulation of cancer cells found in tumour that exhibiting stem cell-like characteristic may be responsible in metastasis of cancer cells as reviewed by Brabletz et al. (2005). Apart from their role in metastasis, many studies have also postulated the theory of tumours being maintained by this small subpopulation of stem-cell like cancer cells that undergo asymmetric cell divisions to produce the differentiated daughter cells, thus forming major proportions of the tumours (Caussinus & Gonzalez, 2005; Clevers, 2005). Over the years, many names have been given to define this stem cell-like subpopulation where "cancer stem cells" (CSCs) is currently the most widely recognised term used for this subpopulation of cancer cells.

Breast CSCs was first discovered in 2003, characterised by the expression of clusters of differentiation (CD) cell surface markers $CD24^{-/low}/CD44^{+}$ to distinguish CSCs from the non-CSCs. As shown by Al-Hajj et al. (2003), as low as 100 transplanted CSCs were capable to generate tumour whereas as high as 10,000 non-CSCs failed to do so. This indicates that the cells expressing $CD24^{-/low}/CD44^{+}$ were highly tumorigenic, which is one of the essential characteristics of CSCs. Not only that, many studies have subsequently reported that breast cancer cell with $CD24^{-/low}/CD44^{+}$ phenotype were associated with drug resistance, metastasis, cancer relapse, which could be the factors of poor prognosis in breast cancer patients (Liu et al., 2010; O'Toole et al., 2011; Wang et al., 2018; Zhao et al., 2016).

Besides, enriched aldehyde dehydrogenase 1 (ALDH1) activity was also observed in breast CSCs. Assessment of CSCs characteristic via ALDH1 was first discovered by Ginestier et al. (2007), who have successfully isolated breast CSCs via ALDH1 assay. Similar to

$CD24^{-/low}/CD44^{+}$ expressing cells, more recent studies showed that elevated ALDH1A1 activity was correlated to tumorigenesis, drug resistance and occurrence of metastasis in breast cancer (Althobiti et al., 2020; Ciccone et al., 2018; Z. Yang et al., 2019).

Later, additional breast CSCs biomarkers including CD133 and CD49f were also identified, (Meyer et al., 2010; M. H. Wright et al., 2008). However, $CD24^{-/low}/CD44^{+}$ phenotype was reported as the predominant biomarkers for breast CSCs among other biomarkers (Ghebeh et al., 2013), and therefore selected for breast CSCs isolation in this study.

2.3.2 Characterisation of breast cancer stem cell

Despite its low number in a tumour, CSCs were found to be one of the causal factors of cancer relapse in breast cancer (Ayob & Ramasamy, 2018; Guo, 2014). CSCs possessed several essential characteristics that are similar to normal stem cells such as self-renewable, infinite proliferative potential, slow replication, elevated DNA repair mechanism and is able to give rise to daughter cells with differentiation potential through asymmetric cell division (Wicha et al., 2006). However, unlike normal stem cells, these stemness-related characteristics are dysregulated in CSCs, driving malignant proliferation, metastasis and tumour recurrence in cancer (Lathia & Liu, 2017; S. Li & Li, 2014).

Cancer relapse mainly rely on two disrupted stem cell characteristics, self-renewability and capability to produce daughter cells with differentiation ability in CSCs, causing regeneration of malignant tumour (B. Bao et al., 2013). Spheroid formation assays are a popular and convenient way to assess self-renewability of malignant stem cells. For instance, a previous study demonstrated that $CD44^{high}$ CSC-like colon cancer cells was capable of generating spheroid with 50% higher spheroid formation efficiency than that of non-CSC-like $CD44^{low}$ colon cancer cells (Ohata et al., 2012). In these assays, malignant stem cells are stimulated to proliferate by addition of epidermal growth factor (EGF) and basic fibroblast growth factor (FGF) to culture media in low adherence tissue culture dishes for cells to generate sphere-like structure (Nguyen et al., 2012; Reynolds & Weiss, 1992).

Spheroid formation assays can be used to evaluate self-renewability of malignant stem cells including brain, colon, melanoma, prostate and breast CSCs (Lombardo et al., 2015; Nguyen et al., 2012). In agreement with spheroid formation assay, early breast progenitor/stem cells with higher self-renewability displayed higher capability to generate 'mammosphere' (Dontu et al., 2003; Dontu et al. 2004).

Study showed significantly low number (< 100 cells) of $CD24^{-/low}/CD44^{+}$ and $ALDH^{+}$ expressing stem-like breast cancer cells were capable to initiate tumour growth but 10000 breast cancer cells without these markers failed to generate tumour in mice model (Al-Hajj et al., 2003; Ginestier et al., 2007). Furthermore, isolated $CD24^{-/low}/CD44^{+}$ cancer cells from lung metastases originated from breast cancer were more tumorigenic as only this population could produce tumours that can be serially passaged (Liu et al., 2010). Studies have consistently demonstrated higher self-renewability in $CD24^{-/low}/CD44^{+}$ characterised breast CSC although the correlation of these phenotypic markers and acquired self-renewability is yet to be defined.

Next, many studies postulated that presence of CSCs is one of the factors contribute to drug resistance which remained as the biggest obstacle before cancer can be fully eradicated. CSCs possess slower cell cycle kinetics, higher capacity to repair DNA (Zhang et al., 2010), enriched anti-apoptotic proteins (Wang & Scadden, 2015), drug efflux transporters (K M Britton et al., 2012; Hirschmann-Jax et al., 2004; Patrawala et al., 2005) and detoxifying enzymes (Honoki et al., 2010). These features can induce resistance to chemotherapeutics and radiotherapy, leading to tumour recurrence and poor survival rate in cancer (Ayob & Ramasamy, 2018; Ginestier et al., 2007).

Elevated level of intracellular reactive oxygen species (ROS) triggered by anti-cancer treatment can induce apoptosis of tumour cells (Liou & Storz, 2010). However, breast CSCs expressing $CD24^{-}/CD44^{+}$ were capable to reduce level of intracellular ROS, thus hindered apoptosis induced by ionizing radiation in breast cancer. Moreover, higher detoxification by counteracting with ROS accumulation was reported in $CD24^{-/low}/CD44^{+}$-expressing breast CSCs, mediated by elevated reduced GSH synthesis and enhanced nicotinamide adenine

dinucleotide (NADH) and flavin adenine dinucleotide (FADH2) products generated from metabolism alteration (Wang et al., 2018). Besides, $ALDH^+$ breast CSCs were found to express lower level of intrinsic ROS as compared to $ALDH^-$ breast cancer cells, suggesting lower susceptibility to anti-cancer treatment in $ALDH^+$ breast cancer cells (Yang et al., 2019).

Although with high proliferative ability, breast CSCs often enter dormancy and mostly remain in quiescent state, therefore have slower growth rate as compared to normal breast cancer cell (Chen et al., 2016). Slower growth rate of breast CSCs allows them to escape from most of the chemotherapy drugs and radiation treatment that exert their effect on rapidly dividing cancer cells (Zou et al., 2011).

Higher expression of ATP-binding cassette (ABC) transporters is associated with higher tolerance to treatment due to higher rate of drug efflux out from cancer cells, causing lower intracellular drug concentration in cancer cells (Sun et al., 2012). Inefficient treatment for breast CSCs could be induced by elevated ABC transporters such as multi drug resistance gene (MDR1) and breast cancer resistance protein (BCRP), which conferred multidrug resistance to the cancer cells (Dean, 2009; Gottesman et al., 2002; Staud & Pavek, 2005). Other than that, breast CSCs are equipped with more efficient mechanism of DNA damage response which leads to lower rate of apoptosis and higher activation of DNA damage checkpoints genes compared to non-CSCs (Chang et al., 2015).

These features of breast CSCs could eventually contribute to higher resistance to anticancer treatment, reducing the efficacy of anti-cancer treatment on breast CSCs.

Followed by that, spreading of cancer cell from primary tumour to surrounding tissues or even distant organs involves a process called metastasis, a primary cause of mortality in cancer patients. It occurs when cancer cells accumulated sufficient strength to detach from primary tumour, intravasate into either circulatory or lymphatic system to travel to desire site, evade from immune attack, extravasate to desire capillary beds, invade and proliferate in the distant organs to generate secondary tumour (Seyfried & Huysentruyt, 2013). Only cancer cells that could survive through migration in circulation and generate new local lesions via extravasation

are capable to complete metastasis. Accumulating evidences demonstrated the CSCs could play a grave role in metastasis (Aktas et al., 2009; Theodoropoulos et al., 2010). In fact, many studies shown positive correlation between the stem cell markers for breast CSCs and metastasis. For example, the enriched CD44 surface marker in breast CSCs was found to mediate epithelial-to-mesenchymal transition (EMT), a crucial process for metastasis in tumour (Xu et al., 2015). EMT is an indispensable step in metastasis to induce migration and invasion in cancer cells (Felipe Lima et al., 2016; Tsai & Yang, 2013). Based on previous studies, EMT can be activated by the binding of hyaluronic acid (HA) to CD44, leading to cancer cell metastasis (Chanmee et al., 2015). On the other hand, inhibition of HA synthesis resulted in lower rate of EMT and metastasis (Arai et al., 2011; T. Saito et al., 2013; Yoshihara et al., 2005).

In human breast tumour, the invasive tumour cells were found to be significantly enriched with $CD24^{-}/CD44^{+}$-expressing cells (Huiping Liu et al., 2010). $CD24^{-}/CD44^{+}$-expressing cells in breast cancer cell lines demonstrated invasive properties and upregulated invasion correlated genes encoding for interleukin (IL)-1, IL-6, IL-8, C-X-C chemokine receptor type 4 (CXCR4), matrix metalloproteinase-1 (MMP-1), and urokinase-type plasminogen activator (uPA) (Sheridan et al., 2006). High percentage of $CD24^{-}/CD44^{+}$-expressing cancer cell in breast primary tumour was suggested to favour distant osseous metastasis (Abraham et al., 2005). Later, study found that up to 71% of tumour cells in early bone marrow metastases from breast cancer expressing $CD24^{-}/CD44^{+}$ phenotype (Balic et al., 2006). Another study conducted by Liu et al. (2010) demonstrated that breast CSCs isolated via $CD24^{-}/CD44^{+}$ phenotype from TNBC patients were highly metastatic. This study was conducted using tumour xenograft models where mammary fat pad implantation of isolated CSCs from TNBC patients produced spontaneous lung metastasis in 100% (73/73) of mice (Liu et al., 2010). These studies did not only display high metastatic ability in breast CSCs but also the contribution of their phenotypic characteristics to the activation of metastasis.

2.3.3 Signalling pathways associated with breast cancer stem cells

Stem cells are regulated by self-renewable associated signalling pathways including janus kinase/signal transducer and activator of transcription (JAK/STAT), NOTCH, Wnt/β-catenin and Hedgehog (Hh) (Crabtree & Miele, 2018; Nalla et al., 2019). These pathways are strictly regulated in normal stem cells for expansion of healthy tissue. However, the same pathways were found to be highly dysregulated in CSCs, causing higher resistance to systemic chemotherapy and induction of new tumour formation that results in cancer relapse (Crabtree & Miele, 2018).

Activation of JAK/STAT signalling pathway is triggered by various ligands, such as interleukins, interferons, hormones, and growth factors when bind to their respective receptors (Rane & Reddy, 2000; Stine & Matunis, 2013). Binding of ligand initiates receptor oligomerization and aggregation of JAK proteins to the cytoplasmic domain of the receptor. Activated JAK proteins through phosphorylation after aggregation then promote phosphorylation of cytoplasmic domain of the receptor and recruitment of STAT proteins. STAT proteins are phosphorylated upon binding with JAK, resulting in dimerization and translocated into nucleus for induction of transcription of target genes (**Figure 2.4**).

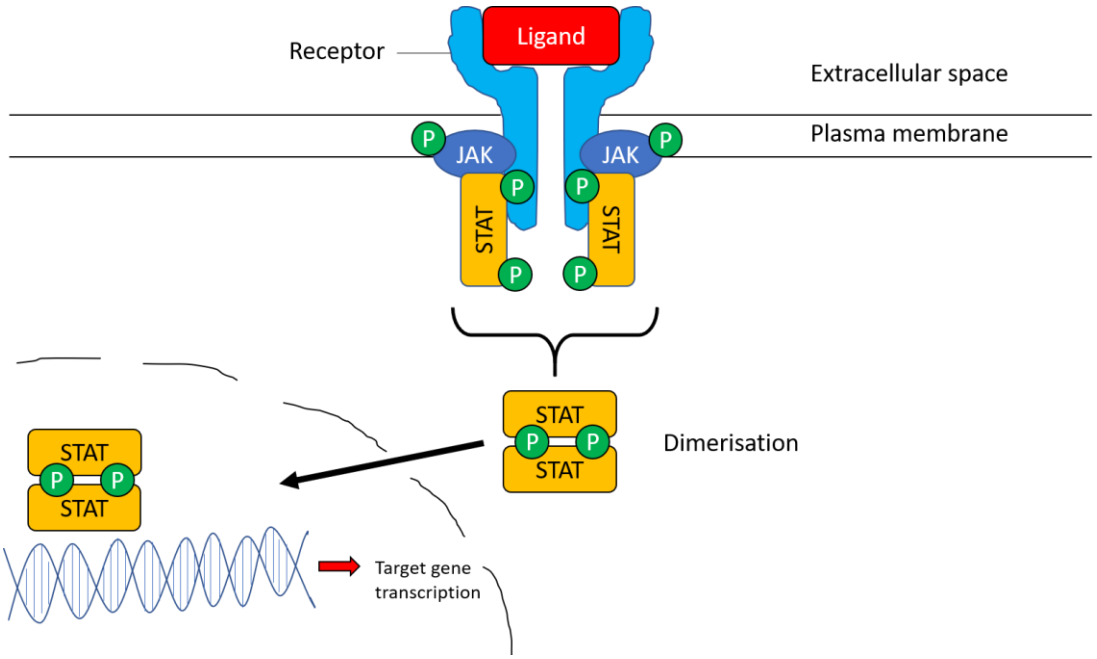


Figure 2.4: JAK/STAT signalling pathway. Activation of JAK/STAT signalling pathway is triggered by its ligands, results in dimerization of STAT consequently induces transcription of target genes.

Dysregulation of JAK/STAT signalling pathway in CSCs can be activated by cytokines, growth factor and hormones from different cells involved in the tumour microenvironment, chemotherapy-induced interleukins and several other proteins. As the result, activation of this pathway contributes to regulation of maintenance and self-renewability in CSCs (Nalla et al., 2019; Thiagarajan et al., 2017; Zhou et al., 2007). Furthermore, JAK/STAT3 pathway was found to mediate tamoxifen resistance in breast CSCs (Marotta et al., 2011; Wang et al., 2012) and involved in metastasis associated by CSCs (Fang et al., 2011; Nalla et al., 2019; Peng et al., 2016).

Next, Notch pathway is also found to correlate with breast CSCs. Activation of Notch pathway involves delta-like (DLL) and jagged (JAG) Notch ligands (DLL1, DLL3, DLL4, JAG1, and JAG2) and its receptors (Notch1-4), where both are transmembrane proteins (Karamboulas & Ailles, 2013). Notch pathway is initiated through the interaction of ligand and receptor expressed in 2 adjacent cells respectively (Karamboulas & Ailles, 2013; Takebe et al., 2015). This interaction then induces proteolytic cleavage of the cytoplasmic domain of the receptor through disintegrin metalloproteases (ADAMs) followed by γ-secretase. Dual cleavage releases

Notch intracellular domain (NICD) into cytoplasm and translocated into nucleus to activate transcription of target genes via the C promoter binding factor 1 (CBF1), suppressor of hairless, LAG-1/recombination signal binding protein for immunoglobulin k J region, also known as CSL/RBPJ transcription factor (**Figure 2.5**).

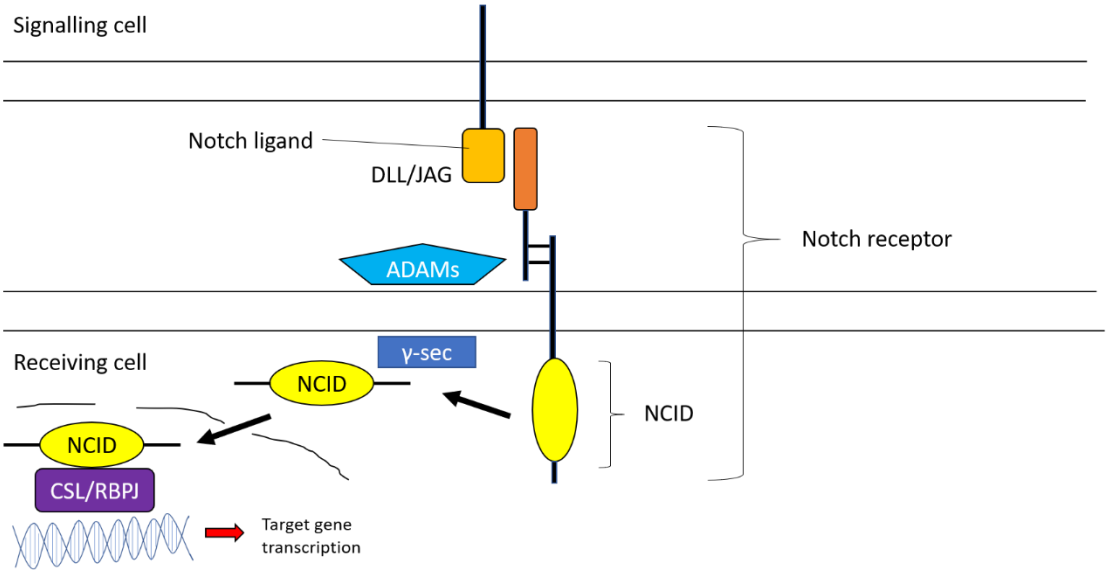


Figure 2.5: Notch signalling pathway. Activation of Notch signalling pathway is triggered by interaction of transmembrane ligand of signalling cell and transmembrane receptor of receiving cells that consequently induces transcription of target genes.

In normal stem cells, Notch signalling pathway is responsible to regulate their self-renewability and differentiate potential stringently, such that they can replenish the stem cell pool while continuing to produce the differentiated daughter cells that are essential for tissue function (J. Liu et al., 2010). The self-renewal or differentiation decisions must be carefully controlled by Notch pathway where its unusual hyperactivation was reported to be involved in cancer development (Reedijk, 2012).

Cancer relapse is one of the outcomes caused by dysregulated stem cell pool expansion and maintenance of CSCs associated to aberrant Notch signalling pathway (Barnawi et al., 2016; Gonzalez et al., 2014; Lagadec et al., 2013). Inhibition of Notch either pharmacologically or genetically led to reduction of breast CSCs population and lower rate of mammosphere

formation in breast cancer cell lines, primary cells, and mouse models of breast cancer (D'Angelo et al., 2015; Grudzien et al., 2009; Simmons et al., 2012). Besides, Notch1 expression was found to positively regulate metastasis and ALDH1 levels in breast CSCs via activation of Notch signalling pathway (Zhong et al., 2016). On the other hand, inhibition of notch receptor induced apoptosis and growth arrest in breast CSCs (Suman et al., 2013). Furthermore, Notch signalling inhibitor hindered γ-secretase and exerted inhibitory effect on metastasis and self-renewability of breast cancer xenograft model (Zhang et al., 2012). A number of EMT-correlated factors including Snail, Slug, and E-cadherin were found to be regulated by Notch (Espinoza et al., 2013). These evidences imply the role of notch signalling pathway in self-renewability and metastasis of breast CSCs.

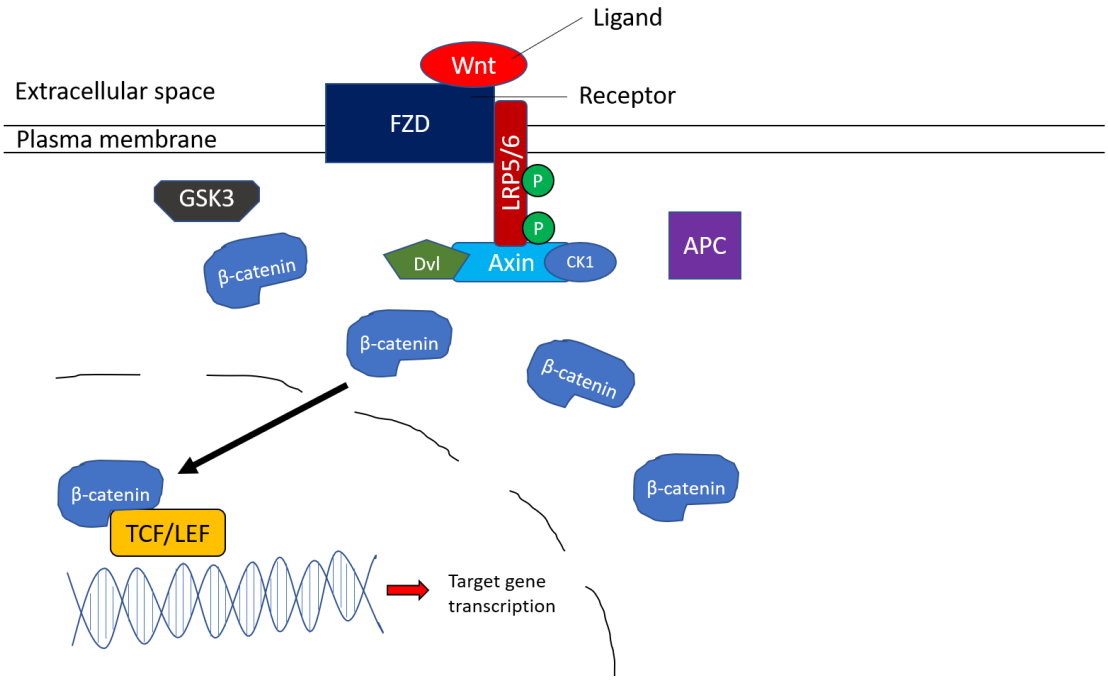
On the other hand, Wnt signalling pathway is associated with tumorigenesis of breast CSCs. Wnt signalling pathway is comprised of canonical (mediated through the transcriptional regulator β-catenin) and non-canonical (β-catenin-independent) Wnt pathways (Kahn, 2014) (**Figure 2.6**). Moreover, canonical Wnt pathway encompassed a 'classical' and new model. In 'classical' model, when secreted Wnt ligand bind to Frizzled (FZD) receptors, activated FZD trigger binding of Dishevelled (Dvl) and phosphorylation of the low-density lipoprotein-related protein (LRP) 5 and LRP 6 coreceptors on a neighbouring cell (Matsui, 2016) (**Figure 2.6A(i)**). Wnt signalling is activated after phosphorylation of one or more cytoplasmic motifs of LRP5/6. However, Wnt pathway can even be activated by single phosphorylated motif (Tamai et al., 2004). Phosphorylated LRP5/6 interact with Axin to destabilise β-catenin destruction complex, which Axin is used as a scaffold and contains Dvl, the serine–threonine kinases, casein kinase 1α/β (CK1), glycogen synthase kinase 3α/β (GSK3) and adenomatous polyposis coli (APC) (Clevers, 2006). Phosphorylation of β-catenin is inhibited when destruction complex is destabilised, causing accumulation of β-catenin in cytoplasm followed by translocation into nucleus. Upon entering nucleus, β-catenin binds to T-cell factor/lymphoid enhancer factor (TCF/LEF) transcription factors to initiate transcription of target genes (Roose et al., 1998). As for new model, destruction complex can be stabilised by Axin in both presence and absence of

Wnt ligand (**Figure 2.6A(ii)**). However, β-catenin is first degraded through phosphorylation-mediated recognition by β-Transducin Repeat-Containing Protein (β-TrCP) in the intact complex, inducing newly synthesised β-catenin to accumulate in cytoplasm before translocating into nucleus and activate transcription of target gene (Azzolin et al., 2012, Azzolin et al. 2014; Li et al., 2012).

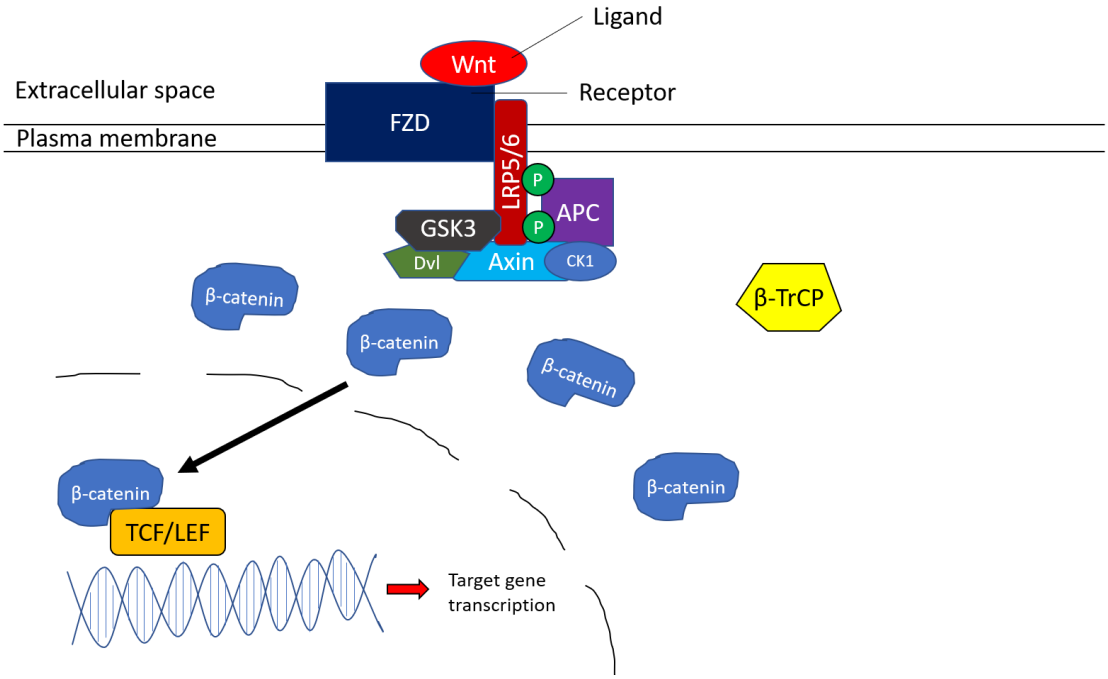
On the other hand, non-canonical Wnt pathway activation occurs independently from β-catenin (**Figure 2.6B**). Binding of Wnt ligand, Wnt5a to FZR receptors triggers interaction of Dvl with Rac1 (Li et al., 1999) and Dvl-associated activator of morphogenesis 1 (DAAM1) separately. Rac1 activates JNK while DAAM1 activates Rho and subsequently Rho-associated kinase (ROCK) to induce the expression of their target genes that are correlated to regulation of cell polarity (Habas et al., 2001; Li et al., 1999).

Alternatively, binding of Wnt5a to FZR interact with G proteins and Dvl (Wong et al., 2003) to activate cyclic guanosine monophosphate (cGMP)-specific phosphodiesterase or phospholipase C (PLC), resulting in a release of intracellular calcium. With that, the downstream signalling pathways, such as protein kinase C (PKC), calcineurin and calcium/calmodulin-dependent protein kinase II (CaMKII) are activated to induce regulation of genes associated with cell adhesion and migration (De, 2011; Dejmek et al., 2006).

A(i): β-catenin dependent Wnt pathway (classical)



A(ii): β-catenin dependent Wnt pathway (new)



B: β-catenin independent Wnt pathway

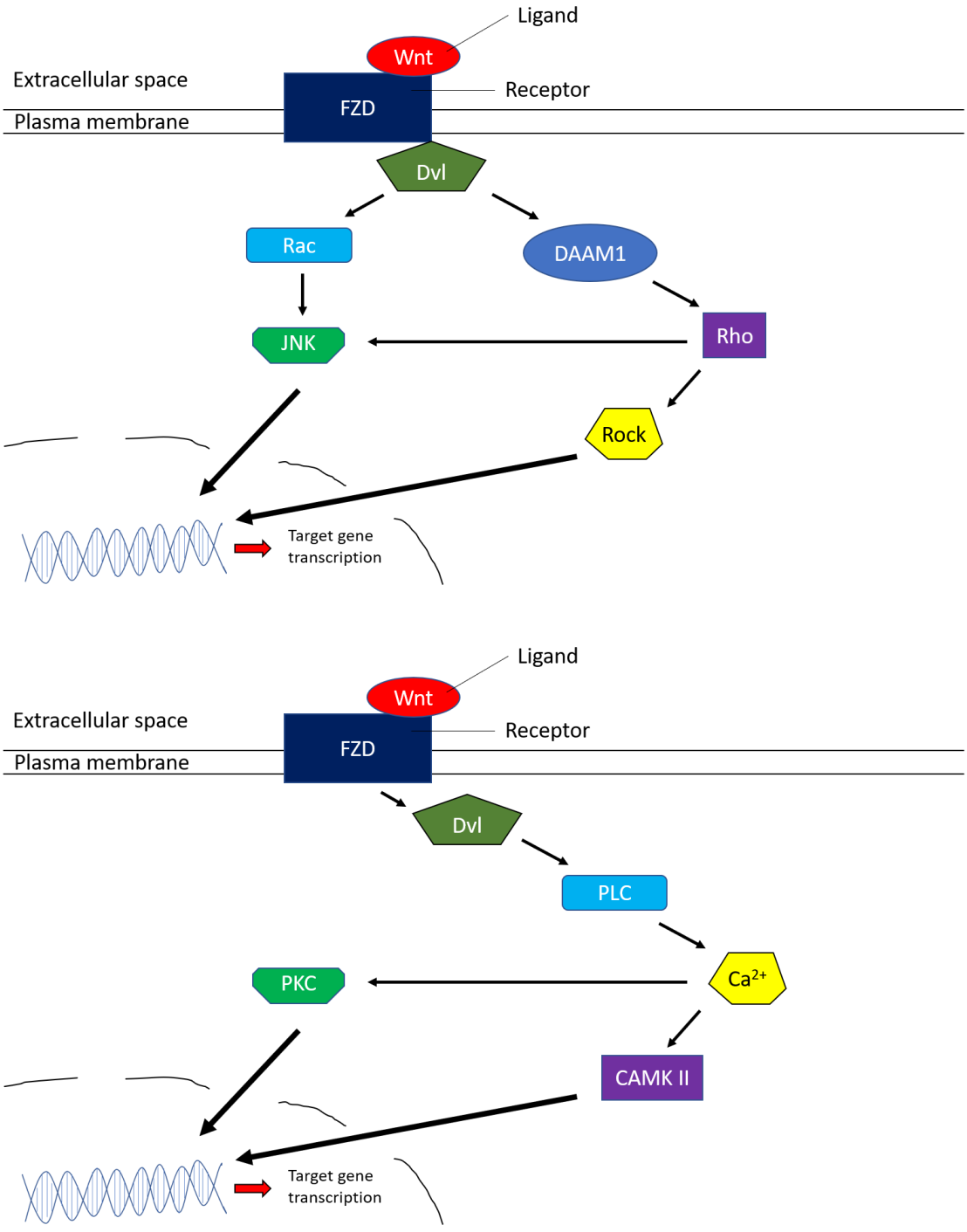


Figure 2.6: Wnt signalling pathway. Wnt signalling pathway can be activated via (A) β-catenin dependent pathway through i) classical and ii) new models and (B) β-catenin independent pathway that consequently induces transcription of respective target genes.

In general, canonical Wnt pathway regulates proliferation, survival, and cell fate decisions while the non-canonical Wnt pathway regulates asymmetrical cell division, cell polarity, and migration. However, these pathways are not mutually exclusive and cross-talk may occur between the two arms (Kahn, 2014).

In normal stem cells, Wnt signaling pathway is responsible for fate determination of cell during embryonic development (Takebe et al., 2011). In cancer cells, Wnt signalling pathway plays an important role in self-renewability and maintenance of CSCs (Mohammed et al., 2016; Zhao et al., 2014). Knockdown of Wnt1 in breast CSCs hindered the expression of CSC-correlated genes including CD44, ALDH1, and Sca-1, reduced tumour-sphere formation and CSCs subpopulation of breast cancer cells (Jang et al., 2015b). Not only that, Wnt signalling pathway is associated with drug resistance and metastasis in breast CSCs (Deshmukh et al., 2017; Jang et al., 2015; Monteiro et al., 2014).

Lastly, Hh signalling pathway is one of the contributors for the aggressiveness of breast CSCs. Activation of Hh pathway can be triggered by three secreted ligands, such as Sonic Hh (SHH), Indian Hh (IHH), and Desert Hh (DHH) through cognate receptor Patched (PTCH) mediated by transmembrane-protein, Smoothened (SMO). Besides, Hh signalling pathway is also affected by an intact primary cilium, a microtubule-containing organelle projecting from the cell surface where its disruption abrogates the signalling cascade. When Hh ligands bind to PTCH receptor, PTCH suppression of SMO is exempted, then initiates SMO ciliary translocation and activation (Habib & O'Shaughnessy, 2016). Freed SMO is activated to promote trafficking of suppressor of fused-glioma-associated oncogene homolog (SUFU-GLI) complexes to the distal cilium followed by dissociation of GLI proteins from SUFU. Formation of GLI transcription factors in their activator form (GLIA) occurred via activation of GLI1 and inhibition of GLI2 and GLI3 proteolytic process. GLIA is then translocated into nucleus to activate transcription of target genes (**Figure 2.7**).

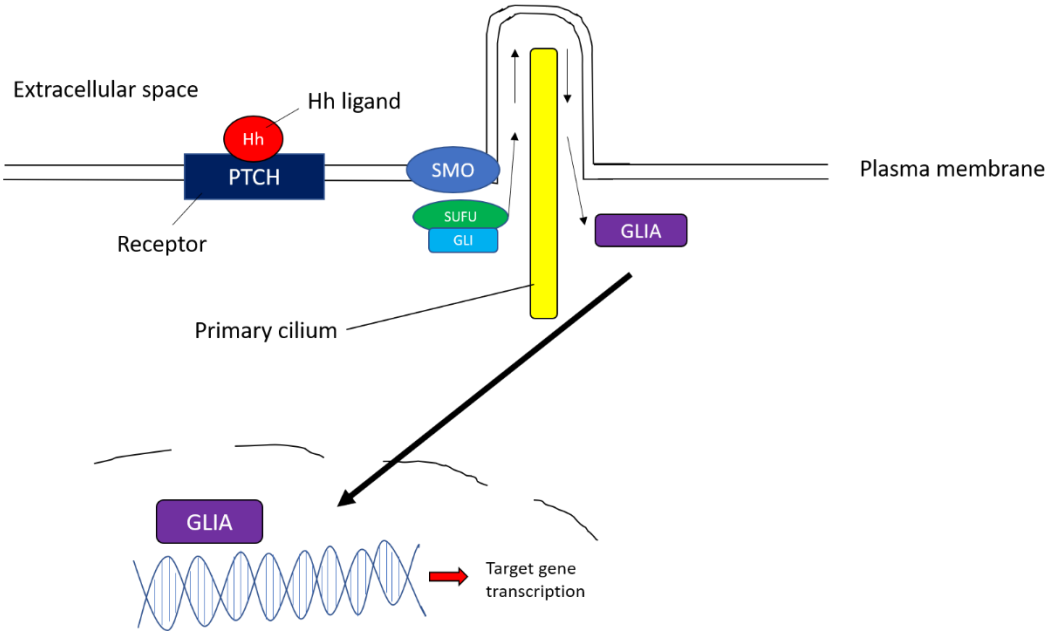


Figure 2.7: Hh signalling pathway. Activation of Hh signalling pathway is triggered by ligand that binds to PTCH receptor to exempt PTCH suppression on SMO to initiate SMO-promoted trafficking of SUFU-GLI complex, leading to formation of GLIA and consequently induces transcription of target genes.

In normal stem cell, Hh pathway is essential for regulation of developmental processes such as cell differentiation and cell proliferation (Jia et al., 2015). As one of key signalling pathways in CSCs, Hh pathway is able to drive the progression of cancers by regulating cancer cell proliferation, malignancy, metastasis and the expansion of CSCs (Sari et al., 2018). Besides, Hh signalling pathway in $CD24^{-}/CD44^{+}$-expressing breast cancer cells was found to associate with poor prognosis in breast cancer patients (O'Toole et al., 2011; Zhao et al., 2016).

In summary, self-renewable breast CSCs and their association with the aforementioned signalling pathways are suggested to play a pivotal role in occurrence of metastasis and drug resistance, leading to cancer relapse in breast cancer.

2.4 Exosome

2.4.1 Biogenesis and secretion of exosome

Exosome is a nano-sized (10-200nm) endosome-derived vesicle (Val et al., 2017) secreted by different types of cell into various body fluids such as urine (Nilsson et al., 2009), plasma (Orozco et al., 2009), saliva (Michael et al., 2010), breast milk (Admyre et al., 2007), brochoalveolar lavage liquid (Prado et al., 2008), and amniotic fluid (Keller et al., 2007). The membrane of exosome is made up by lipid bilayer, encapsulating its cargoes and being secreted from the cell into the extracellular environment via exocytosis. Interestingly, exosome was first discovered in differentiating reticulocytes and suggested to function as a cellular garbage disposal in the process (Johnstone, 1992; Johnstone et al., 1987; Pan et al., 1985). However, information transferred by exosome was later found to orchestrate crucial roles in modulation of physiological functions in cells. Evidences showed that contents of exosome including DNA, ribonucleic acid (RNA), microRNAs (miRNAs) and proteins (Kahlert & Kalluri, 2013) can be transferred to and received by its target cells to induce change of behavioural of the recipient cell (Valadi et al., 2007).

Lipid bilayer membrane structures of exosomes consists of transmembrane proteins and receptors, such as the transmembrane proteins, membrane trafficking proteins (annexins and Rabs proteins), adhesion molecules, lipid raft associated protein, tetraspanins and immuno regulator molecules, encapsulating cytoskeleton proteins, HSPs, metabolic enzymes and multivesicular body (MVB) formation-associated proteins (**Figure 2.8**). The cargoes of exosomes such as proteins, DNA and RNA species can be transferred from the original cell to their target cells (Jia et al., 2017).

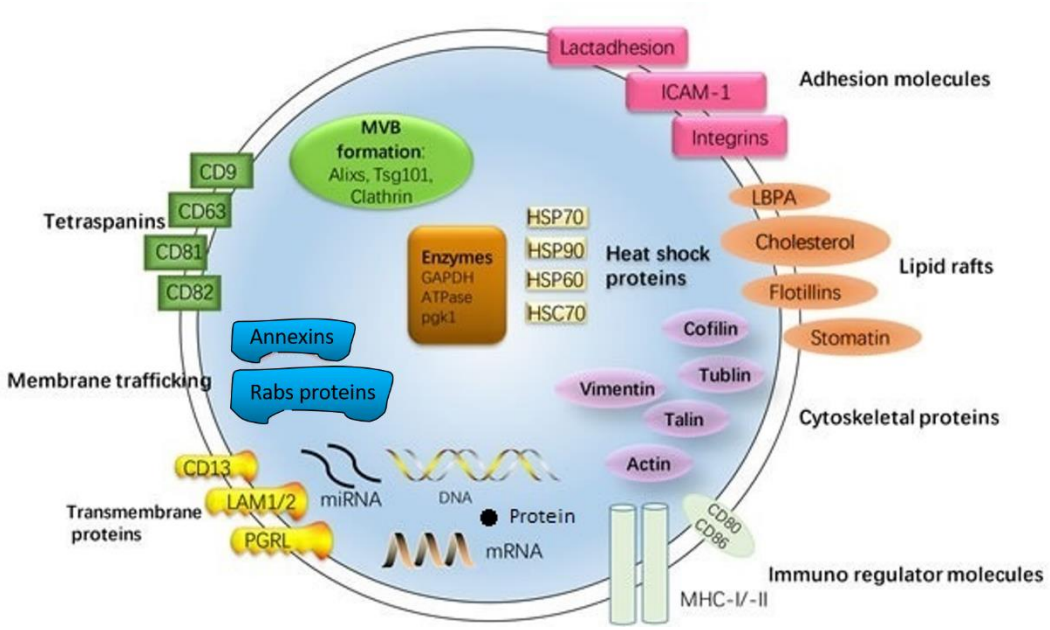


Figure 2.8: Structure and main components of exosomes. Adapted from Jia et al. (2017)**.** Exosome is structured by transmembrane proteins (PGRL, LAMP1/2, CD13), membrane trafficking proteins (annexins and Rabs proteins), adhesion molecules (ICAM-1, Lactadhesion and intergrin), the lipid raft associated protein (Flotillins, LBPA, Cholesterol, stomatin) and tetraspanins (CD9, CD82, CD63 and CD81). Exosomal membranes surface is assembled with immuno regulator molecules, such as MHC-I and -II, CD80 and CD86. Lumen of exosome comprises of other proteins including cytoskeleton proteins (Vimentin, Cofilin, Tublin, Talin, Actin), HSPs, metabolic enzymes (GAPDH, ATPase, pgk1) and MVB formation-associated proteins (Alixs, Tsg101, Clathrin). Abbreviations: p120 catenin, radixin, and immunoglobulin superfamily member 8 (PGRL); lysosomal-associated membrane protein 1/2 (LAMP1/2); Ras-like small GTPase family (Rabs); Heat shock proteins (HSPs); intercellular adhesion molecule-1 (ICAM-1); lysobisphosphatidic acid (LBPA); major histocompatibility complex (MHC); tumour susceptibility gene 101 (tsg101); glyceraldehyde 3-phosphate dehydrogenase (GAPDH) and phosphoglycerate kinase 1 (pgk1).

Exosomes are generated via inward budding of the MVB membrane in the form of intraluminal vesicles (ILVs). They are then released into extracellular space via exocytosis. Two major mechanisms are involved in the production of exosome, one with endosomal sorting complex required for transport (ESCRT) while another without ESCRT. Production of exosomes via ESCRT-dependent pathway requires a set of ubiquitinated proteins including ESCRT 0, I, II, and III and various tetraspanins, namely, CD9, CD63, CD81 and CD82 (Hessvik & Llorente, 2018). Alternatively, exosomes are produced via ESCRT-independent pathway which involves other components such as sphingolipid, ceramide, and the enzyme neutral, sphingomyelinase, which is the enzyme to convert sphingomyelin to ceramide. However, these mechanisms might overlap with each other to work synergistically for exosome production and secretion (Maas et al., 2017).

During secretion process, exosomes will first be trafficked near to cell membrane and secreted upon fusion with the cell membrane. This process can be stimulated by different cellular condition or external stimuli including chemical, environmental and mechanical stimuli, such as gamma-irradiation, hypoxia, low pH (Parolini et al., 2009), and presence of growth factors (Dreyer & Baur, 2016; Graner et al., 2007). Presence of membrane trafficking proteins, adhesion molecules, lipid raft associated proteins, immune regulator molecules enable stimuli to be detected, resulting in release of exosome to extracellular space (Jia et al., 2017). Furthermore, amount of exosome secretion was found to be regulated by a few ESCRT proteins. For example, reduction of ESCRT-0 associated proteins Hrs and TSG101 as well as ESCRT-1 associated protein, namely, signalling transducing adaptor molecule 1 (STAM1) hindered secretion of exosome. Inversely, knockdown of ESCRT-3 and its associated proteins such as charged multivesicular body protein 4c (CHMP4C), vacuolar protein sorting-associated protein 4B (VPS4B), vacuolar protein sorting-associated protein (VTA1) and ALIX induced more exosome secretion (Gross et al., 2012; Hoshino et al., 2013; Tamai et al., 2010). Aside from that, study showed that ALIX could affect protein composition in exosome suggesting its role of loading desirable content into the exosome for secretion (Hessvik & Llorente, 2018).

2.4.2 Tumour derived exosomes (TDEs)

A decade ago, studies displayed enriched secretion of exosome from cancer patient as compared to normal individual (Logozzi et al., 2009; Taylor & Gercel-Taylor, 2008). These TDEs can facilitate cell-cell communication through the transport of growth factors, chemokines, miRNAs, and other small molecules (Akers et al., 2013; Mao et al., 2018). When under stress conditions such as in hypoxia, cancer cells were shown to release more exosomes into their microenvironment that promoted cancer progression (King et al., 2012). In support of the role of exosome as mediator of communication in cancer, Suetsugu et al. (2013) discovered green fluorescent protein (GFP)-labelled exosomes can be transferred from cancer cells to other cells in culture system. Furthermore, *in vivo* study displayed that GFP-exosomes were secreted into

tumour-surrounding tissue and circulate from primary tumours to metastatic niche (Suetsugu et al., 2013). Not only that, study found shuttle of invasion-potentiating miRNAs via exosomes secreted by macrophages into breast cancer cells (Yang et al., 2011).

The ability of exosomes to shuttle miRNAs from donor cells can disrupt gene expression and protein production to induce aberration of biological function in recipient cells, which may result in activation of various processes involved in tumour development and progression. These showed that exosomes are not only transferable to distant site, but also play a grave role in cancer progression via transmission of miRNAs cargo. Therefore, deciphering miRNAs profile of TDEs is of great interest to investigate the information transferred between cancer cells which may contribute to cancer progression**.**

2.5 Micro-RNA

2.5.1 Discovery and biosynthesis of micro-RNAs

The first miRNA, lin-4 RNAs in *C. elegans* was discovered by Rosalind Lee, Rhonda Feinbaum and Victor Ambros (Lee et al., 1993). This single chain RNA molecule which does not encode for proteins has a length of 22 nucleotide (nt), relatively shorter than other RNA species. However, lin-4 RNA was not known as miRNA when it was first discovered in 1993. Followed by the discovery of another RNA with same length of 22nt, which was let-7 from *C. elegans* (Reinhart et al., 2000; Slack et al., 2000), both lin-4 and let-7 RNA were initially known as small temporal RNA (stRNA) due to their overlapping role in controlling the timing of development transitions. Later, researchers observed that many newly identified 22nt RNAs shown no correlation to the timing of development transition, rather were more likely to correlate with their respective cell line. Therefore, the term miRNA was used to refer to the stRNAs and all the other tiny 22nt RNAs with similar features which functions remained unknown (Lagos-Quintana et al., 2001; Lau et al., 2001; Lee & Ambros, 2001).

Biogenesis of miRNA started in nucleus while formation of mature miRNA occurs in cytoplasm (**Figure 2.9**). The DNA encoding for miRNA gene are first transcribed by RNA polymerase II/III (pol II/III) to generate the primary transcripts (pri-miRNAs). RNA polymerase II is the major polymerase type for transcription of miRNAs, though there are small groups of miRNAs associated with Alu elements that are transcribed by RNA polymerase III (Borchert et al., 2006; Schanen & Li, 2011). In nucleus, generated primary transcripts (pri-miRNAs) typically consists of several thousand nt in length with local stem loop structures and a poly-A tail (Lee et al., 2004; Schanen & Li, 2011) while precursor transcripts (pre-miRNAs) are approximately 70 nt in length with hairpin form (Han et al., 2004; Roth et al., 2013). In cytoplasm, pre-miRNAs that is transported out from nucleus are cleaved by another RNase III enzyme known as Dicer along with transactivation response RNA binding protein (TRBP), resulting in mature miRNA/miRNA* duplex with approximately 20 nt (Chendrimada et al., 2005; Pong & Gullerova, 2018; Yi et al., 2003; Yoda et al., 2010). The miRNA duplexes are then incorporated into Argonaute (Ago), aided by the Dicer-TRBP complex to form the RNA-induced silencing complex (RISC) (Pong & Gullerova, 2018; Yoda et al., 2010). RNA helicases then separated or unwound miRNA duplexes into guide strand (mature miRNA) that remains bound to RISC, while passenger strand (miRNA*) proceeds to degradation (Yoda et al., 2010). The Ago protein-bound mature miRNA is subsequently assembled into an effector complex known as the miRNA-containing RNA-induced silencing complex (miRISC) (Loh et al., 2019; Yoda et al., 2010) that eventually inhibits expression of the bound mRNA target.

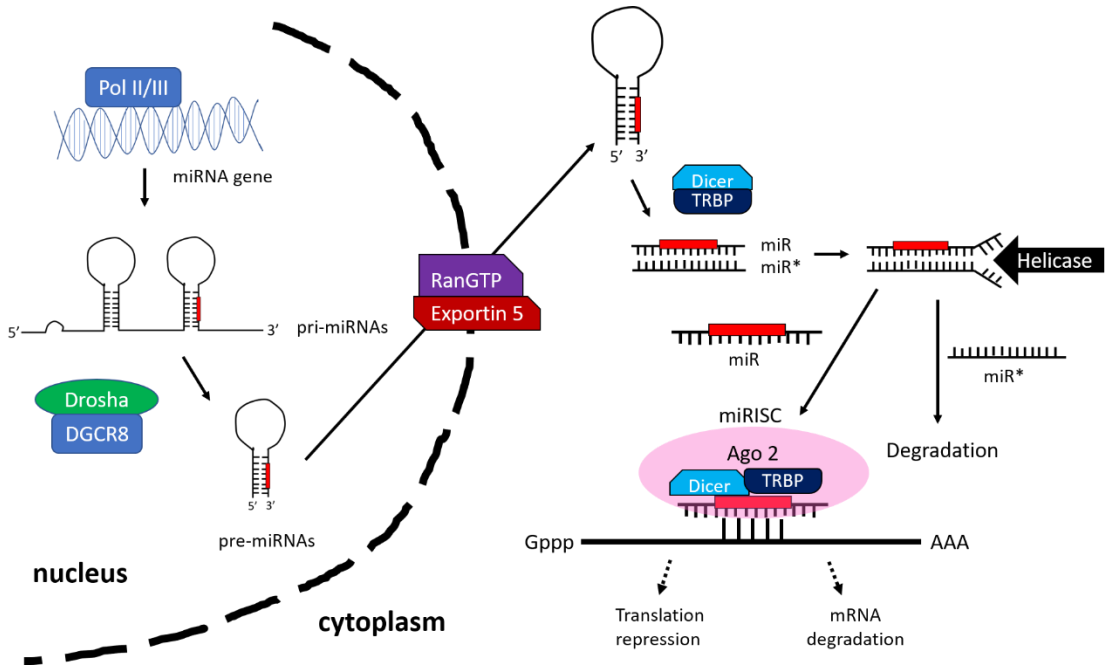


Figure 2.9: Biosynthesis of micro-RNA. In nucleus, miRNA gene encoded DNA are first transcribed by RNA polymerase II/III (pol II/III) to generate the primary transcripts (pri-miRNAs). Followed by transcription, pri-miRNAs processed by a microprocessor complex, Drosha-DiGeorge syndrome critical region gene 8 (Drosha-DGCR8), into the precursor transcripts (pre-miRNAs) and transported from the nucleus into cytoplasm through RanGTP-dependent nuclear transport reporter, exportin 5 and RanGTP to undergo loop-cleavage by another RNase III enzyme known as Dicer along with TRBP. As the result, mature miRNA/miRNA* duplex is generated which then incorporated into Ago with the aid of Dicer-TRBP complex to form RISC. The miRNA duplexes are then separated or unwound into two single strands by RNA helicases which the guide strand (mature miRNA) remains bound to RISC, while passenger strand (miRNA*) proceeds to degradation. The Ago protein-bound mature miRNA is subsequently assembled into an effector complex namely miRISC, once bound to its mRNA target, mature miRNA represses or degrades mRNA based on their degree of complementarity.

Mature miRNAs typically inhibit gene expression by binding to the 3’ untranslated region (UTR) of the messenger RNA (mRNA) (Kehl et al., 2017). The inhibitory mechanism of miRNAs toward its mRNA target is dependent on the degree of their complementarity. Once incorporated into a cytoplasmic miRISC, mRNA with perfect complementarity results in cleavage of mRNA (Fuchs Wightman et al., 2018) whereas partial complementarity between miRNAs and their mRNA targets which is more commonly observed, induced protein translational repression or inhibition (Fuchs Wightman et al., 2018). As the binding site between miRNAs and mRNA is short (8-base seed region on miRNA) (Kehl et al., 2017), a single miRNA has more than one mRNA targets and can regulate their functions in multiple pathways (Bartel, 2009; Melo & Esteller, 2011). Not only that, single mRNA can be

cooperatively targeted and regulated by several different miRNAs (Bartel, 2009; Melo & Esteller, 2011).

2.5.2 Micro-RNAs and cancer

Since discovery of miRNAs in 1993, their biological functions in human cancer were not appreciated until 2002 when study showed that miR-15 and miR-16 which located at chromosome 13q14, found deleted in more than half of chronic lymphocytic leukemias (CLL) and was downregulated in approximately 68% of CLL cases examined. Subsequently, they discovered the hindered miR-15a and miR-16-1 in CLL cases played a role as tumour suppressors that regulate anti-apoptotic proteins such as Bcl-2 and Myeloid cell leukemia 1 (MCL-1) (Calin et al., 2002; Cimmino et al., 2005; Vincent et al., 2014).

Followed by that, many other researchers have identified dysregulated miRNA profiles in different types of human cancers including breast cancer, and demonstrated their roles in promoting or inhibiting breast cancer pathogenesis (Vincent et al., 2014). For example, Pogribny et al. (2010) demonstrated a set of miRNAs were significantly dysregulated in MCF-7 cells resistant to cisplatin. These differentially expressed miRNAs are involved in the control of cell signalling, cell survival, DNA methylation and invasiveness. Besides, study showed that miR-21 was highly overexpressed in breast tumours compared to the matched normal breast tissues among 157 human miRNAs analysed. Overexpression of miR-21 was correlated to increased cell proliferation and reduced apoptosis via modulation of anti-apoptotic protein, bcl-2 (Si et al., 2007). Another study also showed that miR-21 promoted breast cancer proliferation and metastasis by targeting leucine zipper transcription factor like 1 (LZTFL1) (Wang et al., 2019).

Besides, Fan et al. (2014) displayed tumour suppressive role of miR-17-5p in breast cancer metastasis via inhibition of pro-metastatic gene. Oppositely, inhibition of miR-17-5p rendered cells metastatic properties and enhanced lung metastasis from orthotopic xenografts. Supporting their finding, miR-17-5p was found to negatively regulated expression of ETS

variant 1 (ETV1), which is responsible for cell proliferation, invasion, migration in TNBC cells and correlated to poor prognosis in TNBC patients (Li et al., 2017). Interestingly, accumulated studies demonstrated distinctly regulated miRNAs including suppressed miR-200 cluster, miR-183 cluster, let-7, miR-600, miR-30 and enhanced miR-221-222 cluster, miR-142, miR-214 and miR-31 are required for regulation of proliferation, tumour initiation, differentiation, cell stemness, apoptosis, metastasis as well as to govern fate decision in breast CSCs (El Helou et al., 2017; Lv et al., 2017; Shimono et al., 2015; Yu et al., 2010).

Not only are they present in exosome, miRNAs are closely association with breast cancer progression, thus prompted deeper exploration of exomiRNAs for both its carcinogenic effect and as biomarkers for breast cancer.

2.5.3 Formation of exosomal-miRNAs

Mature miRNAs in cytoplasm are first fused with MVB and released into extracellular space, forming exosome containing miRNAs, known as exomiRNAs. There a few postulated sorting mechanisms of miRNAs into exosome. In the miRISC-related pathway, GW182 (a cytoplasmic foci involved in the post-transcriptional regulation of eukaryotic gene expression) and Ago2 in association with miRISC, is sorted into MVBs for secretion and/or lysosomal degradation. This process could promote continuous assembly or disassembly of membrane-associated miRISCs, which is correlated to miRNA loading to exosome (Gibbings et al., 2009). Next, sequence motif of miRNA and guide proteins correlated to exosomal-miRNAs sorting was first discovered by Villarroya-Beltri and colleagues. They found presence of GGAG motif at 3' end of 75% of exosomal-miRNAs (Villarroya-Beltri et al., 2013) whereby, site-directed mutagenesis to this motif in the predominantly exosomal miR-601 resulted in lower level of miR-601 in exosome. Not only that, mutagenesis of the predominantly cellular miR-17 by including this motif, led to increase of miR-17 level in exosome. Furthermore, previous study showed that heterogeneous nuclear ribonucleoprotein (hnRNP) A2B1 and hnRNP-Q bind selectively to miRNAs bearing respective GGAG or GGCU motifs, suggesting that there is sequence-specific miRNA sorting into exosomes (Santangelo et al., 2016). Moreover, study showed the exomiRNAs content can

also be regulated by ceramide (Kosaka et al., 2010). Neutral sphingomyelinase-2 (nMase-2) was found positively regulating biosynthesis of ceramide in HEK293 cells. Knockdown of nMase-2 decreased concentration of exomiRNAs while enhanced nMase-2 increased concentration of exomiRNAs. However, cellular miRNA levels was not affected by nMase-2, demonstrating the role of ceramide specifically in miRNA sorting into exosome (Cha et al., 2015; Challagundla et al., 2015). Lastly, the small GTPase transductor protein, namely, Ki-ras2 Kirsten rat sarcoma viral oncogene homolog (KRAS) has been shown to play a role in miRNA sorting into exosomes where exosomes from mutant KRAS colorectal cancer cells show a distinct miRNA profile compared to wild type cells (Cha et al., 2015).

MiRNAs were found enriched and actively sorted into TDEs (Villarroya-Beltri et al., 2013). In fact, evidences showed that certain miRNAs were enriched in TDEs relative to cells, indicating active sorting of miRNA into exosomes (Hessvik et al., 2012; Pigati et al., 2010). In support of this, respective exosomal and intracellular miRNA profiles within each head and neck carcinoma cell type were shown to be clearly distinct. Thus, this demonstrated that the loading of exosomal cargo is an active and highly regulated process, instead of just a passive process that merely projects intracellular miRNAs content (Langevin et al., 2017). These evidences do not only display enrichment of miRNAs in TDEs, but also showed that miRNAs are selectively sorted into exosomes.

2.5.4 Association of exosomal-miRNA to breast cancer progression

Previous studies demonstrated fairly consistent results between cell line exosome characterisation and plasma exomiRNAs detection in patient derived xenografts (PDX) of mice and breast cancer patients. Their finding was in agreement with a massive genomic and proteomic study which indicated that breast cancer cell lines are reasonable models for analysing context-dependent gene expression, including miRNA expression (Marcotte et al., 2016). Together, these studies showed that cell line study is one of the reliable approaches to investigate the expression of exomiRNAs in breast cancer study (Hannafon et al., 2016). The information transmitted by exomiRNAs in breast cancer cell lines were unravelled to

understand the contribution of dysregulated exomiRNAs in breast cancer tumorigenesis such as drug resistance, metastasis and cell proliferation (**Table 2.1**).

Table 2.1: Biological function of exosomal-miRNAs in breast cancer progression

Exosomal-miRNA(s)	Subtype of breast cancer	Regulation	Biological activity	Gene target(s)	Reference
miR-221, miR-222	MCF7 (ER+)	Upregulated	Drug resistance (Tamoxifen)	ERα, P27	(Wei et al., 2014)
miR-17, miR-30a, miR-100, miR-222		Upregulated	Drug resistance (adriamycin, docetaxel)		(Chen et al., 2014)
miR-29a, miR-30a, miR-100, miR-196a, miR-222		Upregulated	Drug resistance (adriamycin, docetaxel)	PTEN	(Zhong et al., 2013)
miR-20a, miR-23a, miR-24, miR-149, miR-222		Upregulated	Drug resistance (adriamycin)		(Mao et al., 2016)
miR-29a		Upregulated	Drug resistance (adriamycin)	PTEN, GSK3β	(Shen et al., 2016)
miR-222		Upregulated	Drug resistance (adriamycin)	PTEN, AKT, FOXO1	(Shen et al., 2017; Yu et al., 2016)

miR-138-5p, miR-139-5p, miR-197-3p, miR-210-3p, miR-423-5p, miR-574-3p, miR-744-5p, miR-3178, miR-4258, miR-4443, miR-6780b-3p		Upregulated	Drug resistance (epirubicin)		(Zhong et al., 2016)
miR-138-5p, miR-140-3p, miR-210-3p, miR-3613-5p	MDA-MB-231 (TN)	Upregulated	Drug resistance (vinorelbine)		(Zhong et al., 2016)
miR-149-3p, miR-423-5p, miR-671-5p, miR-1246, miR-1268a, miR-4298, miR-4438, miR-4644, miR-7107-5p, miR-7847-3p		Upregulated	Drug resistance (docetaxel)		(Zhong et al., 2016)
miR-128	MCF-7 (ER+)	Upregulated	Proliferation	BAX	(Wei et al., 2016)

miR-141, miR-200a, miR-200b, miR-200c, miR-429	4TO7, 4TO1E (*in vitro, in vivo* Allograft)	Upregulated	Metastatic potential		(Le et al., 2014)
miR-9-5p, miR-195-5p, and miR-203a-3p	MDA-MB-231 (TN), MCF7 (ER+), BT474 (ER+)	Upregulated	Self-renewal and drug resistance (docetaxel/doxorubicin)	ONECUT2	(Shen et al., 2019)
miR-17/20 cluster	MCF-7 (ER+)	Upregulated	Inhibits migration and invasion	IL-8 and CXCL1	(Yu et al., 2010)
miR-10b	MDA-MB-231 (TN)	Upregulated	Invasion	HOXD10 and KLF4	(Singh et al., 2014)

Abbreviation: Oestrogen receptor positive (ER+); Triple negative (TN); Oestrogen receptor alpha (ERα); Cyclin-Dependent Kinase Inhibitor 1B (P27); Prime Time Entertainment Network (PTEN); Glycogen synthase kinase 3 beta (GSK3β); Protein kinase B (AKT); Forkhead box protein O1 (FOXO1); B-cell lymphoma 2 Associated X (BAX); Signal Transducer And Activator Of Transcription 5B (STAT5B); Heat shock proteins 9 (HSP9); B-cell lymphoma 2 (BCL-2); One Cut Homeobox 2 (ONECUT2); Interleukin 8 (IL-8); C-X-C Motif Chemokine Ligand 1 (CXCL1); Homeobox D10 (HOXD10); Kruppel-like factor 4 (KLF4)

Studies demonstrated exomiRNAs secreted by drug-resistant cancer cells enhanced drug tolerance of non-resistant cancer cells (Chen et al., 2014; Mao et al., 2016; Shen et al., 2016, 2017; Wei et al., 2014; Yu et al., 2016; Zhong et al., 2013, 2016) whereas exomiRNAs secreted by breast cancer cells treated with sub lethal dose of chemotherapeutic agents stimulated CSC-like phenotype on recipient cells, which rendered cancer cells to be resistant to therapy (Shen et al., 2019). On the other hand, exomiRNAs secreted by metastatic breast cancer cells induced metastatic ability of non-metastatic tumour cells (Le et al., 2014; Singh et al., 2014). Inversely, exomiRNAs secreted by non-invasive breast cancer cells was capable to hinder migratory ability and invasiveness of the aggressive MDA-MB-231 (Yu et al., 2010). Not only that, previous study demonstrated promotive effect of exomiRNAs on cell proliferation of breast cancer cells (Wei et al., 2016) (**Table 2.1**). Together, these evidences showed the role of exomiRNAs in regulating breast cancer progression.

2.5.5 Exosomal-miRNAs as biomarker for breast cancer

Studies discovered the suitability of miRNAs to be used as biomarker in cancer study. For example, miRNAs expression profiles were found as more accurate marker to classify tumour types than using gene expression profiles (Lu et al., 2005). A study facilitated by support vector machine-based classifier, to categorise early and advance stage breast cancer patient via miRNA expression profiles, was the first to utilise intracellular miRNA profiles to predict breast cancer stage, thus displayed its potential as biomarker (Yerukala Sathipati & Ho, 2018). However, dysregulation of intracellular miRNAs that reside inside the cells are more difficult to access from the cells in deep tissues. Alternatively, many studies have demonstrated the use of exosome-encapsulated circulating miRNAs as potential biomarkers for breast cancer (**Table 2.2**). In fact, there are advantages of utilising exomiRNAs as biomarker relative to intracellular miRNAs as exomiRNAs are not only protected from exposure to nucleases, shear stresses such as high or low pH, they are also stable under long-term storage, and up to 10 cycles of freeze-

thaw process (Chen et al., 2008; Wang et al., 2009). Besides, circulating exomiRNAs were stable and could be reproducibly extracted and assayed in either serum or plasma (Mitchell et al., 2008). The serum exomiRNAs were stable even after drawing blood, which was very important for a reliable biomarker (Chen et al., 2008). Their presence in body fluids, such as peripheral blood and breast milk, making them as more accessible biomarker candidates for disease detection. A number of exomiRNAs had been evaluated for their potential as diagnostic, prognostic or predictive biomarkers in breast cancer (**Table 2.2**).

Table 2.2: Preclinical evaluation of exosomal-miRNAs as biomarkers for breast cancer

miRNA Cargo	Patients cohorts	Source of exosome	Platform used	Reference
Potential biomarkers for breast cancer patient compared to healthy control				
miR-195	83	Blood	RT-qPCR	(Heneghan et al., 2010)
miR-21, miR-106a, miR-155, miR-126, miR-199a and miR-335	68	Serum	RT-qPCR	(F. Wang et al., 2010)
miR-20a, miR-21 and miR-214	168	Serum	RT-qPCR	(Schwarzenbach et al., 2012)
miR-1, miR-92a, miR-133a and miR-133b	164	Serum	Microarray, RT-qPCR	(Chan et al., 2013)
miR-182	46	Serum	RT-qPCR	(P.-Y. Wang et al., 2013)
miR-148b and miR-133a	137	Plasma	RT-qPCR	(J. Shen et al., 2014)
miR-138	83	Blood	Microarray, RT-qPCR	(Waters et al., 2014)
miR-505-5p, miR-125b-5p, miR-21-5p and miR-96-5p	197	Plasma	RT-qPCR	(Matamala et al., 2015)

let-7c	90	Serum	RT-qPCR	(X.-X. Li et al., 2015)
miR-148b-3p, miR-652-3p and miR-10b-5p	87	Serum	ddPCR	(Mangolini et al., 2015)
miR-21 and miR-1246	16	Plasma	Small RNA sequencing, RT-qPCR	(Hannafon et al., 2016)
miR-4270, miR- -1225-5p, miR-188-5p, miR-1202, miR-4281, miR-1207-5p, miR-642b-3p, miR-1290 and miR-3141	46	Serum, Plasma	Microarray, RT-qPCR	(Hamam et al., 2016)
miR-1246, miR-1307-3p, miR-4634, miR-6861-5p and miR- -6875-5p	1280	Serum	Microarray, RT-qPCR	(Shimomura et al., 2016)

Plasma (miR-106a-3p, miR-106a-5p, miR-20b-5p, and miR-92a-2-5p); serum (miR-106a-5p, miR-19b-3p, miR-20b-5p, and miR-92a-3p)	64	Serum, Plasma	RT-qPCR	(M. Li et al., 2018)
Potential biomarkers for early stage of breast cancer				
miR-127-3p, miR-148b, miR-409-3p, miR-652 and miR-801	247	Plasma	RT-qPCR	(Cuk et al., 2013)
miR-21 and miR-92a	100	Serum	RT-qPCR	(H. Si et al., 2013)
miR-15a, miR-18a, miR-107, miR-133a, miR-139-5p, miR-143, miR-145, miR-365 and miR-425	108	Serum	RT-qPCR	(Kodahl et al., 2014)
miR-484	137	Serum	RT-qPCR	(Zearo et al., 2014)
miR-155, miR-19a, miR-181b and miR-24	63	Serum	RT-qPCR	(Sochor et al., 2014)

miR-155	259	Serum	RT-qPCR	(S. Gao et al., 2017)
Potential biomarkers to differentiate stages or subtypes of breast cancer				
miR-4270, miR-1225-5p, miR-188-5p, miR-1202, miR-4281, miR-1207-5p, miR-642b-3p, miR-1290 and miR-3141	46	Serum, Plasma	Microarray, RT-qPCR	(Hamam et al., 2016)
miR-34a	89	Serum	RT-qPCR	(C. Roth et al., 2010)
miR-34a, miR-373, miR-17	152	Serum	RT-qPCR	(Eichelser et al., 2013)
Potential biomarkers for cancer relapse in breast cancer				
miR-122 and miR-375	68	Serum	Small RNA sequencing, RT-qPCR	(X. Wu et al., 2012)
miR-18b, miR-103, miR-107 and miR-652	130	Serum	RT-qPCR	(Kleivi Sahlberg et al., 2015)

miR-21-5p, miR-375, miR-205-5p, miR-382-5p, miR-376c-3p and miR-411-5p	90	Serum	RT-qPCR	(Huo et al., 2016)
miR-16, miR-30b and miR-93	153	Plasma	Microarray, TaqMan-qPCR	(Ni et al., 2018)
miR-340, miR-17-5p, miR-130a-3p and miR-93-5p	16	Serum	RT-qPCR	(Sueta et al., 2017)
miR-223-3p	27	Plasma	Small RNA sequencing, RT-qPCR	(H. Wu et al., 2020)
Potential biomarkers for drug resistance in breast cancer				
miR-125b	56	Serum	RT-qPCR	(Hongjiang Wang et al., 2012)
miR-122 and miR-375	68	Serum	Small RNA sequencing, RT-qPCR	(X. Wu et al., 2012)

miR-155	103	Serum	RT-qPCR	(Y. Sun et al., 2012)
Potential biomarkers for metastasis in breast cancer				
miR-10b, miR-34a and miR-155	89	Serum	RT-qPCR	(C. Roth et al., 2010)
miR-10b and miR-373	60	Plasma	RT-qPCR	(Weijie Chen et al., 2013)
miR-21 and miR-92a	100	Serum	RT-qPCR	(H. Si et al., 2013)
miR-17, miR-155	152	Serum	RT-qPCR	(Eichelser et al., 2013)
let-7b and miR-202	102	Serum	RT-qPCR	(Joosse et al., 2014)
miR-21 and miR-105	53	Serum	TaqMan-qPCR	(Rodríguez-Martínez et al., 2019)
miR-21	102	Serum	RT-qPCR	(Asaga et al., 2011)

Abbreviation: Ductal carcinoma *in situ* (DCIS); reverse transcriptase-real time polymerase chain reaction (RT-qPCR); TaqMan-real time polymerase chain reaction (TaqMan-qPCR); droplet digital PCR (ddPCR)

Dysregulated exomiRNAs either in blood, plasma or serum of breast cancer patients were evaluated to identify potential biomarkers for breast cancer (**Table 2.2**). Serum and plasma prepared from peripheral blood are easily accessible where serum is the liquid that remains after the blood has coagulated, while plasma is the liquid that remains when clotting of blood is prevented with addition of anticoagulant. Although the choice between serum or plasma samples is rarely justified in publications, previous study showed no differences being observed between plasma and serum particularly from human samples in regards to the expression profile of the most expressed miRNAs, alignment rates and average miRNA detection (Dufourd et al., 2019).

Studies demonstrated that dysregulated exomiRNAs in breast cancer can be used to differentiate breast cancer patients from healthy control (Chan et al., 2013; Hannafon et al., 2016; Heneghan et al., 2010; Li et al., 2018; Li et al., 2015; Mangolini et al., 2015; Matamala et al., 2015; Schwarzenbach et al., 2012; Shen et al., 2014; Wang et al., 2010; Wang et al., 2013; Waters et al., 2014). Moreover, a number of studies showed identifiable dysregulation of circulating exomiRNAs in early stage of breast cancer, indicating their potential to be developed as biomarkers for early detection of breast cancer (Cuk et al., 2013; Gao et al., 2017; Kodahl et al., 2014; Si et al., 2013; Sochor et al., 2014; Zearo et al., 2014). The use of exomiRNAs profile to discriminate breast cancer from other type of cancers was shown by a remarkably large-scale study that profiled exosome from serum of 1280 patients with breast cancer, 2836 healthy controls, 451 from patients with other cancer types and 63 from patients with non-breast benign diseases. This study discovered and validated a panel of five miRNAs (miR-1246, miR-1307-3p, miR-4634, miR-6861-5p and miR-6875-5p) that discriminated between patients with breast cancer and those with other cancer types as well as from healthy controls (Shimomura et al., 2016). Not only that, aberration of circulating miRNAs enabled detection of hormonal receptor status, tumour stage and presence of metastasis in breast cancer. For example, a study reported elevated miR-34a in serum was correlated with tumour stage while increased serum levels of miR-10b, miR-34a and miR-155 were associated with occurrence of metastases in breast cancer (Roth et al., 2010). In consistent to finding by C.

Roth et al. (2010), another study reported correlation of miR-34a and miR-155 to breast cancer (Eichelser et al., 2013). Their study revealed the expression of miR-155 and miR-17 were significantly higher in metastatic breast cancer. Moreover, elevated miR-373 was associated with Her-2-negative primary tumour while miR-17 and miR-34a were correlated with PR/ER status in breast cancer.

More examples have also displayed the potential of circulating-miRNAs as biomarker for occurrence of metastasis in breast cancer. For instance, studies demonstrated the dysregulated circulating exomiRNAs were able to differentiate patients with metastatic breast cancer from those with non-metastatic breast cancer, benign breast cancer and healthy controls (Joosse et al., 2014; Rodríguez-Martínez et al., 2019). Chem and colleague showed the expression of circulating exomiR-21 could be used to distinguish breast cancer patients from healthy controls and further distinguish patients with visceral metastases (Asaga et al., 2011). Not only that, detected dysregulation of circulating exomiRNAs were found to associate with lymph node metastasis in breast cancer (Chen et al., 2013; Kong et al., 2018; Si et al., 2013).

Dysregulated profiles of circulating exomiRNAs may also be associated with breast cancer patients' response to chemotherapy (Sun et al., 2012; Wang et al., 2012; Wu et al., 2012) and occurrence of cancer relapse (Huo et al., 2016; Kleivi Sahlberg et al., 2015; Ni et al., 2018; Sueta et al., 2017; Wu et al., 2012). With these characteristics, studies discovered the potential of circulating exomiRNAs as predictive biomarkers for effectiveness of chemotherapy and possible occurrence of cancer relapse in breast cancer (Kleivi Sahlberg et al., 2015; Wang et al., 2012; Wu et al., 2012) (**Table 2.2**).

Apart from that, modulation of the dysregulated exomiRNAs that are correlated to breast cancer progression had been suggested as a therapeutic strategy for cancer treatment. This can be done either by restoring the tumour suppressive miRNAs through synthetic miRNA mimics or inhibiting oncogenic miRNAs through anti-miRNA oligonucleotides. For example, exosome may be loaded with anti-miRNA oligonucleotides with sequence complementary to the targeted mature oncogenic miRNAs and delivered into the tumour for therapeutic purpose {Formatting Citation}.

In conclusion, from a diagnostic perspective, circulating exomiRNAs extracted from serum, plasma or blood could reflect the activities within breast cancer cells, making them useful for early disease detection, monitoring and prediction of occurrence of metastasis, treatment response and cancer relapse. From a therapeutic perspective, circulating exomiRNAs have been implicated in enhancement or restrained tumour growth, conferred drug resistance and regulation of the induction of metastasis, thereby showing valuable information which could be translated into breast cancer treatment.

2.6 Bioassays

2.6.1 Cancer stem cell-like cell isolation via magnetic activated cell sorting

Magnetic activated cell sorting (MACS) is widely used to isolate different types of cells in various studies (Hansel et al., 1991; B. Schmitz et al., 1994; J. Schmitz et al., 1993; Semple et al., 1993). It is also one of the commonly used methods to isolate stem cell-like cells from cancers (Bussolati et al., 2008; Ricci-Vitiani et al., 2007). Unlike fluorescence activated cell sorting (FACS) method that uses detection of fluorescent labelling and/or scattered light for cell isolation (Herzenberg et al., 2002), MACS method involved small magnetic microbeads conjugated with specific antibodies to isolate the desire subpopulation. Although FACS is capable of isolating multiple subpopulations simultaneously, it requires longer time and more expensive machinery, which is less time- and cost-effective than that of MACS (Sutermaster & Darling, 2019). As the objective of this study focussed on isolating one subpopulation which was $CD24^{-}/CD44^{+}$-expressing CSC-like subpopulation, MACS method was selected to isolate this CSC-like subpopulation from parental MDA.

2.6.2 Immunophenotyping assays

Immunophenotyping is defined as a process that uses antibodies to identify cells based on the types of antigens or markers expressed by the cells. Immunofluorescent technique is now widely used for cell identification, derived from immunophenotyping with conjugation of

fluorochromes to antibodies whereby enable phenotype of cells to be revealed by evaluation of fluorochrome labels. Antigen or markers labelled by fluorochromes can be assessed qualitatively and quantitatively via two detection platforms, namely flow cytometry and fluorescence microscopy.

FACS was invented in the late 1960s by Bonner, Sweet, Hulett, Herzenberg, and others to perform flow cytometry and cell sorting of viable cells (Bonner et al., 1972; Herzenberg et al., 2002; Hulett et al., 1973; Loken et al., 1977). It uses fluorescence as a quantitative tool to measure the expression of conjugated protein quantitatively. Alternatively, expression of conjugated proteins can be observed qualitatively through fluorescence microscope (Drummen, 2012; Masters, 2010).

As FACS-flow cytometry is a sensitive, fast and easy method to analyse a large number of single cells for several parameters simultaneously (Herzenberg et al., 2002; Lopresti et al., 2019) while fluorescence microscope enable visual of protein expression (Rost, 2017; Vikram et al., 2020), combination of these techniques are often used for immunophenotyping in many cancer studies including for breast cancer (Boo et al., 2017; T. Liu et al., 2019; Lopresti et al., 2019). Therefore, both flow cytometry and fluorescence microscope were used to investigate the expression of breast CSCs biomarkers including $CD24^{-}/CD44^{+}$ and ALDH1A1 quantitatively via FACS-flow cytometry and qualitatively via fluorescence microscope in this study.

2.6.3 Assessment of cytotoxicity of cisplatin

2.6.3.1 MTT assay

Cell viability is a universal endpoint that is measured in many drug discovery efforts across a variety of disease models. There are numerous cell viability assays available that measure different biomarkers, including adenosine triphosphate (ATP), reducing potential, and cellular enzyme activity of cells. 3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyl tetrazolium bromide (MTT) is one of the assays used to estimate the metabolic condition of the experimented cells through a quantitative colorimetric-based assay (Oez et al., 1990). This assay was first

discovered in 1983 and functions via impulse that viable cells have practical mitochondria and possess the ability to digest MTT through the aid of enzymes (Mosmann, 1983). NAD(P)H-dependent cellular oxidoreductase enzymes within the mitochondria responsible for changing the yellow water-soluble tetrazolium salt into the water-insoluble purple formazon crystals through a metabolic process is called the mitochondria dehydrogenease (Twentyman & Luscombe, 1987). To dissolve the purple formazon crystals for quantification of the absorbance value, organic solvents namely (DMSO) is used to solubilise the crystals, which the intensity of solution is measured by a wavelength of 500-600 nm. As viable cells are exclusively capable to reduce the tetrazolium salts metabolically, MTT assay is able to distinguish between viable and non-viable cells, making it an exceptionally suitable assay to assess cell viability. In breast cancer research, MTT assay often serves as preliminary screening to determine the effectiveness of drugs or compounds in killing selective breast cancer cell lines (Hastings & Kenealey, 2017; Razak et al., 2019). The advantages of this assay include being inexpensive and reproducible, thus was used to determine IC_{50} of cisplatin for later use on acquirement of cisplatin treated subpopulations in this study, as elaborated in section 3.1.4.

2.6.3.2 Apoptosis assay

Programmed cell death, also known as apoptosis, is identified by specific morphological attributes and systems which are energy-dependent. Activation of apoptosis is characterised by specific morphologic features inclusive of the loss of plasma membrane asymmetry and attachment, plasma membrane blebbing, condensation of the cytoplasm and nucleus, and internucleosomal cleavage of DNA (Cotter et al., 1997; Elmore, 2007).

Annexin V/propidium iodide (Annexin V/PI) is a commonly used assay to assess occurrence of apoptosis based on the specific morphologic features of apoptotic cells. The membrane phospholipid phosphatidylserine (PS) of apoptotic cells in early stage is translocated from the inner to the outer leaflet of the plasma membrane, thereby exposing PS to the external cellular environment (Kenis et al., 2004; Vermes et al., 1995). Annexin V, a 35-36 kDa Ca^{2+}-dependent

phospholipid-binding protein, is highly affinite for PS, thereby binds to exposed apoptotic cell surface PS (Casciola-Rosen et al., 1996; van Engeland et al., 1996; Vermes et al., 1995).

Annexin V can be conjugated to fluorochromes (such as FITC) while retaining its high affinity for PS and thus serves as a sensitive probe for flow cytometry analysis of cells undergoing apoptosis (Elmore, 2007). Consequently, PS translocation precedes the loss of membrane integrity, which accompanies the later stages of apoptosis. Therefore, staining with Annexin V is typically used in conjunction with a vital dye such as PI to differentiate early and late apoptotic cells. As PI is a membrane-impermeant nucleic acid intercalator, viable cells with intact membranes exclude PI, whereas the membranes of dead and damaged cells are permeable to PI. Therefore, cells that are considered viable display Annexin V^-/PI^- while cells that are in early apoptosis display Annexin V^+/PI^-, and cells that are in late apoptosis or already dead display Annexin V^+/PI^+.

Similar to previous studies (Fani et al., 2016; Jiang et al., 2017; X. Zhu et al., 2020), Annexin V/PI analysis via flow cytometry was selected to evaluate apoptotic event triggered by cisplatin quantitatively rather than assessing these signals qualitatively under fluorescent microscope due to higher sensitivity of detecting singular apoptotic cells and distinguish different stages of apoptosis quantitatively.

2.6.3.3 Cell cycle analysis

Cell cycle is defined as “the operation of dividing a single cell into two daughter cells” (Binder & Landman, 2009). The process is complex, involving growing and proliferating of cells in the development of the organism, repairing of damaged DNA, response towards injury including cancer (Alberts et al., 2002).

The use of flow cytometry in cell cycle analysis was first introduced in 60’s (Kamentsky et al., 1965; Van Dilla et al., 1969) and has now become a commonly used assay to assess cell cycle pattern in breast cancer studies (Murad et al., 2016; Ross et al., 2003; Spyratos, 1993). Cell

cycle process involves a sequence of events, morphologically subdivided into four phases namely are G1, S, M and G2 phases (Lim & Kaldis, 2013).

Flow cytometry cell cycle analysis that is capable to determine the cell cycle phase at a single cell level via the expression of DNA content often used to study the alteration of cell cycle pattern in breast cancer cells (J.-Y. Hong et al., 2014; E. Park, 2016). With that, flow cytometry cell cycle analysis is used to detect cell cycle pattern in breast cancer cell induced by cisplatin in this study.

2.6.3.4 Invasion and migration assays

As compared to benign tumour that remains confined to its original location without invading or spreading to distant body sites, malignant tumour can metastasise to distant site by invading surrounding normal tissue and spreading throughout the body (Cooper, 2000). Metastasis is one of the contributors for high mortality rate in cancer patients (Seyfried & Huysentruyt, 2013). Since migratory and invasive capacities are essential to induce metastasis, investigating invasion and migration in tumour cells maintain important factors for anti-cancer studies.

The name Boyden chamber assay was originated by Boyden, whom invented the transwell assay to study the chemotactic influence of leukocytes (Boyden, 1962). Thereafter, the transwell migration and invasion assays were designed using modified and enhanced versions of the Boyden chambers to fit to various cell types including epithelial (Chen et al., 2012) or more specifically breast cells (Tao et al., 2008). The transwell migration assay depends on dual medium including chambers with porous membrane whereby cells transmigrate (**Figure 2.10A**). Migrated cells are then detected and quantified by fixation on the membrane followed by staining and quantification.

On the other hand, the concept of the transwell invasion assay setup is similar to that of transwell migration assay (**Figure 2.10B**). However, cells have to invade through protein barrier gel, for example, Matrigel®, which is secluded from the Englebreth-Holm-Swarm mouse sarcoma, a tumour rich in ECM proteins (Moutasim et al., 2011). Matrigel overlays the

porous filter in advance of cell seeding on top chamber (Albini & Benelli, 2007; Marshall, 2011). The function of the protein gel is to block any non-invasive cells from migrating. In comparison, cells that are capable to invade will degenerate the protein gel and infuse through to attach at the bottom of the filter. Cells that invaded across Matrigel and membrane pores can be stained and quantified.

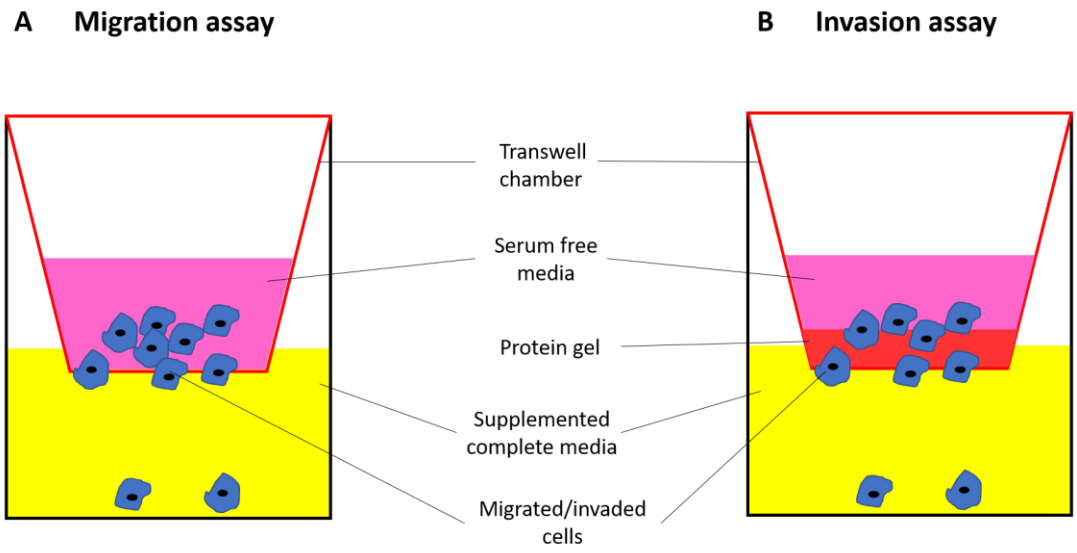


Figure 2.10: Transwell assays. (A) Migration assay and (B) invasion assay.

This assay has been broadly commercialised in the production of disposable transmembrane inserts for 24- or 96-well tissue culture plates. Furthermore, membranes with a variety of pore sizes to accommodate different cell types are available (Chung et al., 2018; Hulkower & Herber, 2011), making this a suitable assay to study metastatic ability in various cell types including breast cancer cell line.

In fact, Transwell migration and invasion assays often used to evaluate the migratory and invasive abilities of breast cancer cells (C.-L. Liu et al., 2019; Tian et al., 2018). Therefore, anti-metastatic effect of cisplatin on breast cancer cells in this study was assessed via transwell migration and invasion assays.

2.6.3.5 Mammosphere formation efficiency test

CSCs are characterised as self-renewal, possessing ability to renew itself indefinitely in an undifferentiated state, unlimited proliferative potential, even capable to give rise to daughter cells with differentiation potential through asymmetric cell division (Wicha et al., 2006). Self-renewability of cells can be evaluated via spheroid formation assay, which was first discovered by Reynolds and Weiss in year 1992 (Reynolds & Weiss, 1992). They aimed to isolate stem cell-like cell from adult brain tissues and culture the single cell in suspension culture with media supplemented with EGF and FGF. To their surprised, neural cell in suspension culture was able to generate a sphere-like structure and they then named it as 'neurosphere'. The generated neurosphere was again being dissociated and culture in media consisting EGF and FGF. Remarkably, the mechanically dissociated primary neurosphere is able to form the anchorage independent secondary sphere indicating acquirement of self-renewability in stem cell (Reynolds & Weiss, 1992). With the same conditioned media, sphere formation assay was developed to investigate the ability of malignant stem cells such as the brain, colon, melanoma prostate and breast CSC in forming the sphere-like structure (Nguyen et al., 2012).

Today, mammomsphere formation assay is one of the most commonly used assays to evaluate self-renewability of breast cancer cells based on its ability to generate sphere-like structure observed under microscope for calculation of sphere forming efficiency (% SFE) derived from both primary tissue and breast cancer cell lines (Lombardo et al., 2015; R. Wang et al., 2014). Therefore, the effect of cisplatin on self-renewability of breast cancer cells was evaluated via mammomsphere formation assay in this study.

2.6.4 Quantitative Real Time Polymerase Chain Reaction

Study of gene expression is a useful tool in quantifying transcription levels of specific genes. In order to determine the gene expression, measurement of mRNA levels of specific genes is important (Padovan-Merhar & Raj, 2013). There are several methods that are commonly used in analysing mRNA expression include northern blotting, *in situ* hybridization, and Real Time

Polymerase Chain Reaction (RT PCR) (Bartlett et al., 2012). Among these methods, Real Time reverse transcription (RT^2) PCR is the most sensitive and reliable method for gene expression analysis. It monitors the amount of amplicon generated as the reaction progresses by monitoring reporter dye fluorescence (Overbergh et al., 2003).

This advanced technology manages to quantitate PCR products in real time, during plateau as well as during exponential phase (Sugden & Winter, 2008). In addition, a simplified and pre-optimised real-time PCR system, namely RT^2 Profiler™ PCR Array System can reduce the time-consumed to study multiple genes simultaneously. RT^2 Profiler™ PCR Array is widely used for gene profiling in many breast cancer studies (Arikawa et al., 2007; Comanescu, 2013). The system is made up of a 96- or 384-well plate containing primers for a set of 84 related genes, plus five housekeeping genes (HKG), and three controls. It combines the quantitative performance of SYBR® Green-based real-time PCR with the multiple gene profiling capabilities of a microarray.

In order to efficiently study multiple genes of interest simultaneously, RT^2 Profiler™ PCR Array was used in this study to evaluate cisplatin-induced dysregulation of these genes in breast cancer cells.

2.6.5 Exosome characterisation

EVs are a category composed of subgroups including exosomes, prostasomes, oncosomes, dexosomes, microparticles, promininosomes, argosomes, and exosome-like vesicles, depending on their cellular origin and biological function (Zaborowski et al., 2015). Among these, the exosome has been reported as a cell communicator in cancer (Camussi et al., 2010; Zaharie et al., 2015). Exosome is often characterised by its size of 10-200 nm (Val et al., 2017) and expression of specific biomarker through transmission electronic microscopy (TEM) (Klymiuk et al., 2019; Mazzeo et al., 2015; Théry et al., 2002) .

Dynamic light scattering (DLS) is an approach used to determine sizes of microvesicles, including exosome (Lane et al., 2015; Val et al., 2017) whereby light-scattering characteristics

of laser light on suspended particles that are undergoing Brownian motion is used to measure particle size (Stetefeld et al., 2016). However, the accuracy of DLS is limited by the purity of sample, such as difficulty in differentiating between EVs in suspensions of particles with varied sizes (Wimmel et al., 2001). As such, DLS is often combined with TEM to characterise exosomes based on size and biomarker expression for better accuracy (Qadir et al., 2018; Val et al., 2017; Zhang et al., 2018).

TEM is a microscopy technique in which a beam of electrons is transmitted through a specimen to form an image. The first TEM was demonstrated by Max Knoll and Ernst Ruska in 1931, with this group developing the first TEM with resolution greater than that of light in 1933 and the first commercial TEM in 1939 (Lambert & Mulvey, 1996).

TEM is the most widely used instrument to monitor the quality and purity of EV-containing samples, because it is able to discriminate single EVs from similar sized non-EV particles (Coumans et al., 2017; Van Deun et al., 2017). Most exosomes were visualised by negative TEM (Liu & Wang, 2011; Thery et al., 2006; Wang et al., 2015), and these results were confirmed by immunolabeling of tetraspanins including CD9, CD82, CD63 and CD81 in whole mount vesicle for characterisation of exosome (Hessvik & Llorente, 2018; Jung & Mun, 2018; Mazzeo et al., 2015).

As refereed to previous studies (Qadir et al., 2018; Val et al., 2017; Zhang et al., 2018), combination of DLS and immune-labelling TEM was used to verify the identity of exosome not only through its size via DLS, also by the expression of its biomarkers via TEM in this study.

2.6.6 Transcriptomic profiling of exosomal-miRNAs

Small RNAs (sRNAs) are known to regulate various organisms in many biological processes including embryo development, cell differentiation, proliferation and apoptosis (Ghildiyal & Zamore, 2009). Eukaryotic regulatory sRNAs typically range in size from ~20 to 30 nt comprises three major classes which are miRNA, siRNA and piwi-interacting RNAs (piRNA).

The synthesis of miRNAs can occur in extracellular microvesicles, results in formation of exomiRNAs. Altered exomiRNAs expression profiles have been implicated in a number of cancers (Ebrahimkhani et al., 2018; Q. Huang et al., 2018), highlighting the importance of understanding the impact of dysregulated exomiRNAs in cancer biology.

Next-generation sequencing (NGS) is one of the most powerful tools for analysis of sRNAs. In advantage, NGS allows for the discovery of novel sRNAs, has a better signal to noise ratio than microarrays and does not suffer of saturation effects as compared to microarray or qPCR. Furthermore, it allows detection of single base differences and has a higher throughput than qPCR (Gao et al., 2016; Ghildiyal & Zamore, 2009). Prior knowledge on miRNAs is not needed in this case. In a typical sRNA library preparation process, adapters are ligated to the RNAs followed by reverse transcription and PCR amplification. The generated sRNA library can be sequenced by different sequencers such as NextSeq, HiSeq, MiSeq and MiniSeq, each with different maximum output. With that, users can choose the most suitable sequencing platform according to the objective of their studies. NGS is commonly used in transcriptomic profiling of miRNAs in breast cancers (Martinez-Gutierrez et al., 2019; Wu et al., 2020).

Other than NGS, a relatively newer technology known as nanostring nCounter miRNA Expression Assay has been introduced to investigate the expression of miRNAs. With total RNA input as low as 100ng, nanostring enables users to rapidly and efficiently profile 798 highly curated human miRNAs. This product provides a sensitive, reproducible, and highly multiplexed method for detecting specific miRNAs within purified RNA isolated from any source, including fresh or frozen cells and tissues, formalin-fixed paraffin-embedded (FFPE) samples and biological fluids, including exomiRNAs. Nanostring offers direct digital counts of each miRNA without the use of reverse transcription or amplification. It is a unique, targeted discovery and validation tool that enables collection of expression data in a short amount of time with minimal hands-on manipulation. Nanostring has now involved in profiling or validating miRNAs expression in various studies including cancer studies (Armstrong et al., 2015; Foye et al., 2017).

Taken together, NGS was used to profile the expression of exomiRNAs where the determined dysregulated exomiRNAs was further validated by Nanostring to identify potential therapeutic targets and biomarkers for breast cancer in this study.

Chapter 3

Method

3.1 Preparation of triple negative breast cancer subpopulations

3.1.1 Cell culture for triple negative breast cancer cells

The human TNBC cell line, MDA-MB-231 was purchased from (ATCC, USA). MDA-MB-231 was cultured under adherent culture condition sustained by RPMI-1640 (Sigma-Aldrich, USA) supplemented with 10% of fetal bovine serum (FBS) (Gibco, USA). Cancer cells were cultured at 37°C in 5% CO_2 humidified atmosphere and were sub-cultured upon reaching 70% confluency.

3.1.2 Isolation of triple negative breast cancer stem cells via magnetic-activated column sorting

Isolation of CSC-like subpopulation was conducted via magnetic-activated cell sorting (MACS) separation which involved MACS Column and MACS separator (Miltenyi Biotec, Germany). The parental MDA-MB-231 cells (abbreviated as pMDA from here onwards) were filtered using 70µm cell strainer (BD, USA) to obtain single cell suspension prior to cell sorting. MACS separation was conducted according to protocol provided by manufacturer. Briefly, the 24^- subpopulation was first isolated CD24 MicroBead kit (Miltenyi Biotec, Germany) via negative selection of CD24 expressing cells followed by positive selection of CD44 expressing cells using anti-CD44-PE (Biolegend, USA) and anti-PE MicroBead (Miltenyi Biotec, Germany) as shown in **Figure 3.1**. This resulted in acquirement of $CD24^-/CD44^+$ CSC-like subpopulation (abbreviated as CSC-MDA from here onwards).

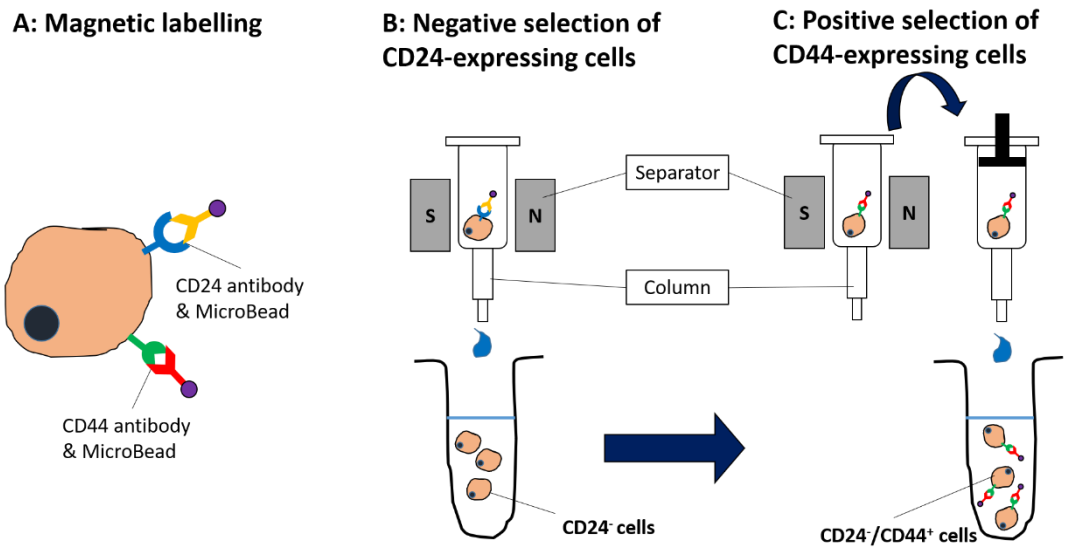


Figure 3.1: Isolation of CSC-like subpopulation, ($CD24^-/CD44^+$)-expressing MDA-MB-231 via MACS. A) Cells of interest are magnetically labelled with respective antibodies and MACS MicroBeads. Labelled cells is separated magnetically, first through B) negative selection of $CD24^-$-expressing cells by collecting the flow-through fraction followed by C) positive selection of $CD44^+$-expressing cells by eluting the retained cells from the column removed from separator to acquire $CD24^-/CD44^+$-expressing cells.

3.1.3 Determination of IC_{50} value of cisplatin on triple negative breast cancer cells via MTT assay

MTT assay (Sigma-Aldrich, USA) was conducted to determine the half maximal inhibitory concentration (IC_{50} value) of cisplatin (Merck, USA). The pMDA cells were seeded at a cell density of 8000 cells/well in flat bottom 96 well plate (BD, USA) and incubated at 37°C in 5% CO_2 for 24 hours to achieve 70% confluency. Seeded cells were treated with a range of cisplatin from 0 - 200µM. Cisplatin treated plate was incubated for 24, 48 and 72 hours followed by addition of 20µL of MTT solution (5mg/mL). The insoluble formazan crystal formed was observed and solubilised in Dimethyl sulfoxide (DMSO) (Fisher Scientific, USA) before absorbance readings were taken by microplate reader (µ Quant Biotek Instruments, USA) at wavelength of 570nm. The shortest time point with stabilised drug cytotoxicity was selected for acquirement of cisplatin-treated triple negative breast cancer subpopulations.

3.1.4 Acquirement of cisplatin-treated triple negative breast cancer subpopulations

A seeding density of 2 x 10^5 of cells from pMDA and MACS-isolated CSC-MDA were seeded in 6 well plate (BD, USA) for 24 hours until cancer cells reached 70% confluency. As longer culturing duration can induce higher rate of cell differentiation, it is crucial to select the shortest time point with stable cisplatin cytotoxic effect, which was the acquired IC_{50} value of cisplatin at 48 hours, to acquire cisplatin-treated subpopulations. This can ensure the best maintenance of cell stemness of the MACS-isolated untreated CSC-MDA after a total of 72 hours of culture duration (including 24 hours of incubation to allow cells adhere on plate followed by 48 hours of incubation duration, similar to treatment duration for cisplatin-treated subpopulations) prior assessing the phenotypic and genotypic characteristics of cancer cells. After 24 hours, the seeded cells that achieved 70-80% of confluency were then treated with the identified IC_{50} value of 56μM cisplatin (Merck, USA) for 48 hours. A total of four MDA subpopulations as listed below were acquired and investigated by bioassays in following sections.

I. pMDA control
II. Treated pMDA
III. CSC-MDA control
IV. Treated CSC-MDA

3.2 Evaluation of breast cancer stem cell biomarkers expression

3.2.1 Immuno-phenotyping for expression of surface markers CD44 and CD24 via flow cytometry

Anti-CD44 and anti-CD24 flow-cytometry was conducted to quantify breast CSC-like cells ($CD24^-/CD44^+$). Cancer cells were harvested and counted to prepare appropriate number of cells for flow analysis. A number of 1 x 10^6 cells/mL of single cell suspension were suspended in 100µL of PBS followed by double staining with 1:40 dilution of anti-CD24-FITC (Biolegend, USA) and anti-CD44-PE (Biolegend, USA) in PBS. The reaction was incubated at 4°C for at least 1 hour in dark. The cells were then washed with PBS to remove unbound dyes. Stained cell pellet was resuspended in 500µL of fresh PBS then subjected by BD Accuri C6 flow cytometer and analysed using BD Accuri C6 software (BD, USA).

3.2.2 Immuno-phenotyping for expression of intracellular protein ALDH1A1 via flow cytometry

As ALDH1A1 is one of the commonly used biomarkers for breast CSCs, anti-ALDH1A1-APC flow cytometry was conducted to study the stemness of MDA subpopulations. Anti-ALDH1A1 (Cell Signalling Technology, USA) antibody was first conjugated to lightning-link APC antibody labelling kit (Innova Biosciences Ltd, USA) to obtain anti-ALDH1A1-APC antibody. The APC-conjugated anti-ALDH1A1 was prepared at a concentration of 0.01µg/µL, mixed in PBS. LL-modifier and LL-APC were first added to anti-ALDH1A1 and incubated at 4°C overnight. LL-quencher was then added and incubated with anti-ALDH1A1-APC at 4°C for 30 minutes in dark according to manufacturer's manual prior usage. Harvested cells were pelleted and fixed by 4% paraformaldehyde solution (Thermo Fisher Scientific, USA) at 4°C for 10 minutes. The fixed cells were washed twice in cold PBS and permeabilised by 100µL of cold 0.25% Triton-X-100 (Merck, USA) with PBS for 10 minutes. The permeabilised cells were rinsed twice with cold PBS (incubated for 5 minutes each time) before stained with 1:200 dilution of anti-ALDH1A1-APC in PBS at 4°C for 1 hour in dark. The cells were washed with

PBS to remove unbound dyes. Stained cells pellets were resuspended in 500µL of fresh PBS then subjected by NovoCyte flow cytometer and analysed using NovoExpress Software (ACEA Biosciences, USA).

3.2.3 Immunofluorescence microscopy detection of surface markers CD44/CD24 and intracellular marker ALDH1A1

Both pMDA and isolated CSC-MDA were seeded at a cell density of 8000 cells/well in flat bottom 96 well plate (BD, USA) and incubated at 37°C in 5% CO_2 for 24 hours to achieve 70% confluency. These seeded cells were treated by 56µM of cisplatin for 48 hours. Before the control and treated pMDA and CSC-MDA were fixed, medium was carefully removed from wells and cells were rinsed twice with PBS to remove residue from medium. Cells in each well were fixed by 50µL of 4% paraformaldehyde solution (Thermo Fisher Scientific, USA) at room temperature for 10 minutes. Fixed cells were then washed twice by PBS at room temperature and blocked with blocking solution, 1% BSA (Sigma-Aldrich, USA) in PBS for 1 hour at room temperature. Cells were double stained with two antibodies, anti-CD24-FITC (Biolegend, USA) and anti-CD44-Alexa-Fluor 647 (Biolegend, USA) with 1:40 dilution of antibodies in PBS at 4°C for 1 hour in dark, allowing conjugation of antibodies to the surface markers on the cells. These immunofluorescent samples for ALDH1A1 were fixed similarly as described early for immunophenotyping of CD44 and CD24 surface markers but without the blocking step. Fixed cells were permeabilised with 50µL of cold 0.25% Triton-X-100 with PBS (Merck, USA) for 10 minutes. The permeabilised cells were rinsed twice with cold PBS (incubated for 5 minutes each time) before stained with 1:200 dilution of anti-ALDH1A1-APC in PBS, at 4°C for 1 hour in dark. Both CD24/CD44 and ALDH1A1 stained cells were rinsed twice with PBS (incubated for 5 minutes each time) before counterstained with 0.5µg/mL of Hoescht solution (Sigma-Aldrich, USA) at room temperature for 10 minutes. The counterstained cells were rinsed once with PBS for 10 minutes and 100µL of fresh PBS was added in well prior to imaging. CD24 and CD44-stained cells were imaged via FITC, Alexa-Fluor 647 and Hoeschst filters while ALDH1A1-stained cells were imaged via APC and Hoeschst filters using Zeiss microscope

(Carl Zeiss AG, Germany). The captured images were further processed and exported via Zeiss Zen microscope software (Carl Zeiss AG, Germany).

3.3 Assessment of cytotoxicity of cisplatin on breast cancer stem cells

3.3.1 FITC-Annexin V/PI staining apoptosis assay

Harvested cells were washed twice with PBS and resuspended in 1 x binding buffer provided in FITC Annexin V Apoptosis Detection Kit I (BD, USA) where 100µL of solution consisting 1 x 10^5 of cells was stained by FITC-Annexin V and PI as described in manufacturer's manual for FITC Annexin V Apoptosis Detection Kit I (BD, USA). The cells were incubated at room temperature for 15 minutes in dark. For identification of apoptotic cells, stained cells were subjected by BD Accuri C6 flow cytometer and analysed using BD Accuri C6 software (BD, USA).

3.3.2 Cell cycle analysis

Harvested cells were washed with buffer solution (BD Biosciences, USA) and subjected to cell cycle analysis using Cycletest™ Plus DNA Reagent Kit (BD Biosciences, USA) as referred to the manufacturer's manual. The washed cell pellets were successively resuspended and incubated in solution A (trypsin), solution B (trypsin inhibitor and RNAse A) and solution C (PI) for 10 minutes respectively. Stained cells in mixed solution were subjected by NovoCyte flow cytometer and analysed using NovoExpress Software (ACEA Biosciences, USA).

3.4 Assessment of metastatic ability of cisplatin-treated breast cancer stem cells

3.4.1 Transwell migration and invasion assays

Migration and invasion assays were conducted to assess the metastatic ability of TNBC cells after cisplatin treatment. For invasion assay, 1×10^5 cells in 500µL of serum free RPMI were seeded onto 8µm Transwell-24 (BD, USA) coated with 50µL of 1:3 dilution of Matrigel basement membrane matrix (BD, USA) in serum free RPMI, which was mounted into a 24 well plate (SPL, Korea). The mounted Transwell-24 was submerged into 750µL of RPMI supplemented with 10% FBS. Migration assay was conducted similarly using Transwell-24 without Matrigel coating. Cells were incubated 72 hours for invasion assay and 48 hours for migration assay followed by cell fixation using methanol (Merck, USA). The dissolved 0.2% (w/v) crystal violet (Sigma-Aldrich, USA) with absolute ethanol (Merck, USA) was used for staining the cells before Transwell was viewed under microscope (Nikon, Japan). Stained Transwells were destained using 30% acetic acid (Thermo Fisher Scientific, USA) to quantify the invaded and/or migrated cells by measuring the absorbance readings at wavelength of 595nm using Elisa plate reader (µ Quant Biotek Instruments, USA). Rate of invasion and migration were calculated based on formula shown below.

$$\text{Rate of migration and invasion (\%)} = \frac{\text{OD tested subpopulation}}{\text{OD pMDA control}} \times 100\%$$

3.5 Evaluation of self-renewability of cisplatin-treated breast cancer stem cells

3.5.1 Mammosphere formation efficiency test

Harvested cells were seeded into U-bottom 96 well plate (BD, USA) at a cell density of 200 cells per well and sustained in DMEM/F12 (Sigma-Aldrich, USA) supplemented with 20ng/µL

of epidermal growth factor (EGF) (Peprotech, Israel), 20ng/µL of basic fibroblast growth factor (bFGF) (Peprotech, Israel), 1% B27 (Gibco, USA), 10µg/mL insulin (Thermo Fisher Scientific, USA), 0.5µg/mL hydrocortisone (Nacalai Tesque, Japan), 1% Penicillin-Streptomycin solution (Gibco, USA), 1% L-glutamine (Thermo Fisher Scientific, USA) and 0.4% Bovine Serum Albumin (BSA) (Sigma-Aldrich, USA). To study the mammosphere formation efficiency of these MDA subpopulations, the seeded cells were observed for 14 days and the numbers of generated mammosphere were counted. Mammosphere formation efficiency was calculated based on formula shown below.

$$\text{Mammosphere formation efficiency (\%)} = \frac{\text{Number of well with generated mammosphere on day 14}}{\text{48 mammosphere seeded well on day 0}} \times 100\%$$

3.6 Profiling of cisplatin-induced dysregulation of cancer stem cells correlated-gene expression

3.6.1 RNA extraction from breast cancer cells

Total RNA extraction was conducted using QIAzol (Qiagen, Germany) with modification to the protocol. 3 x 10^6 of culture cells were prepared for RNA extraction to ensure optimal yield and purity of total RNA is obtained. The collected cells were homogenised in 750µL of QIAzol and incubated at room temperature for 5 minutes. After the incubation, 150 µL of chloroform (Merck, USA) was added to the homogenised sample and shake vigorously for 15 seconds prior to a 3 minutes incubation. Phase separation of the homogenised sample was conducted by centrifuging the sample at 12000 xg for 15 minutes under 4°C. After centrifugation, the mixture was separated into 3 phases. RNA was retained in the uppermost aqueous phase which was being carefully transferred to a new tube for RNA precipitation. 375µL of 2-propanol (Merck, USA) was added to the RNA aqueous and incubated at room temperature for 10 minutes. The mixture was centrifuged at 12000 xg for 10 minutes under 4°C for RNA precipitation.

Supernatant was carefully removed and discarded without disrupting the precipitated white RNA pellet. RNA pellet was washed twice with 750µL of 75% ethanol and the dislodged RNA pellet was re-pelleted by centrifuging at 8600 xg for 5 minutes while 75% ethanol wash was removed and discarded. Lastly, the cleaned and dried RNA pellet was resuspended in 30µL of RNase free water and kept in -80°C until further use. The purity and concentration of extracted total RNA was measured using NanoPhotometer UV/Vis spectrophotometer (Implen, Germany). Only RNA samples that achieved good purity (absorbance ratios between 1.8 to 2.0 for A_{260}/A_{280} and greater than 1.8 for A_{260}/A_{230}) were used in this study.

3.6.2 Quantitative Real-Time PCR array for cancer stem cells-correlated genes

DNA contaminant was cleaned from 2.5µg of total RNA prior to cDNA conversion using RT^2 first stand kit (Qiagen, Germany). The cDNAs obtained were loaded into RT^2 profiler PCR array for human CSCs panel (Qiagen, Germany) followed by addition of SYBR Green Mastermix (Qiagen, Germany). The cDNA was subjected to real-time polymerase chain reaction using CFX96 (Bio-Rad, USA) to quantify gene expression of the CSC-correlated genes in PCR array. Gene expression was analysed using Bio-Rad CFX Manager (Bio-Rad, USA) while the dysregulation of genes was analysed using RT^2 Profiler PCR Array Data Analysis Webportal (Qiagen, Germany) normalised using beta-2-microglobulin (B2M), hypoxanthine phosphoribosyltransferase 1 (HPRT1) and ribosomal protein lateral stalk subunit P0 (RPLP0) housekeeping genes.

3.6.3 Gene ontology enrichment pathway analysis of dysregulated genes

Enrichment of gene ontology (GO) terms of dysregulated gene profiles were determined through PANTHER classification system (http://www.pantherdb.org/) (Thomas et al., 2003). The PANTHER overrepresentation test version 15.0 was used (Fisher's exact test with false discovery rate (FDR) multiple test correction) by comparison with GO database release 2020-02-14. P-values < 0.05 were regarded as statistically significant for the analysis.

3.7 Exosome characterisation

3.7.1 Preparation of cultured media for collection of exosome

Each MDA subpopulation was cultured under adherent culture condition sustained by RPMI-1640 supplemented with 10% of exosome-depleted serum (Gibco, USA). Cancer cells were cultured at 37°C in 5% CO_2 humidified atmosphere. Exosome-free media was collected from untreated cancer cells upon 80% confluency whereas exosome-free media from treated cancer cells were collected after cancer cells were treated with 56μM cisplatin for 48 hours (Merck, USA).

3.7.2 Exosome isolation

Cell debris was removed from the collected media by centrifuging at 10000 xg for 30 minutes at 4°C using Eppendorf centrifuge machine 4810R (Eppendorf, Germany) and filtered using 0.22μm syringe filter membrane (Merck, USA). The exosome in cell debris-fee media was concentrated by ultracentrifugation at 100,000 xg for 90 minutes at 4°C using ultracentrifuge machine (Beackham Coulter, USA). Supernatant was carefully removed while the purified exosome pellet was fixed with 4% paraformaldehyde solution (Thermo Fisher Scientific, USA) for 5 min.

3.7.3 Verification of exosome via dynamic light scattering measurement

DLS measurements were performed with a ZetaSizer 3000-HA (Malvern Instruments, UK). Samples were diluted 1:1000 in PBS to a total volume of 1.5 mL. Three measurements were performed, with standard settings (Refractive Index = 1.37, absorption: k = 0.01, temperature = 25°C).

3.7.4 Verification of exosome via transmission electron microscope

A volume of 7 μL of fixed exosome was loaded on carbon/formvar coated 400 mesh copper EM grids (ProSciTech Pty Ltd, Australia) and incubated for 5 minutes. The exosome-loaded

grid was rinsed with PBS thrice, 10 minutes for each rinse. Grid was blocked with a drop of blocking buffer (PBS containing 1% BSA) for 15 minutes. Grid was stained with 1:25 dilution of anti-CD63 and anti-CD9 mouse monoclonal (Santa Cruz Biotechnology, USA) in 0.1% BSA-PBS respectively 2 hours at 4°C. Grid was rinsed thrice with 0.1% BSA-PBS and stained with anti-mouse secondary antibody 10 nm of gold particle diluted at 1:25 dilution in 0.1% BSA-PBS for 1 hour. The grid was washed by two separate drops of 0.1% BSA-PBS followed by one separate drop of distilled water wash. Negative staining was performed by staining the grid surface with of filtered 1% uranyl acetate (UA) (Thermo Fisher Scientific, USA). Excess UA was removed from grid using filter paper followed by a quick rinse of the grid with water to further remove the excess staining solution. Stained grid was dried for 10 minutes at room temperature and viewed under JEM-2100F transmission electron microscope (Joel, Japan) at 100kV.

3.8 Exosomal-miRNAs profiling with different panels

3.8.1 Isolation of miRNAs from exosome

Exosomal-RNA was extracted from collected exosome-free cultured media. Firstly, cell debris was removed from the collected media by centrifuging at 10000 xg for 30 minutes at 4°C using Eppendorf centrifuge machine 4810R (Eppendorf, Germany) and filtered using 0.22µm syringe filter membrane. The exosome in cell debris-fee media was concentrated by ultracentrifugation at 100,000 xg for 70 minutes at 4°C using ultracentrifuge machine (Beackham Coulter, USA). Exosomal-RNA was extracted from the concentrated media using ExoRNeasy maxi kit (Qiagen, Germany) as described by manufacturer.

3.8.2 Library preparation and screening of exosomal-microRNA via next generation sequencing

For small RNA library preparation, at least 30ng of miRNA was used as input. The libraries were constructed using NEBNext Multiplex Small RNA Library Prep Set for Illumina (New

England BioLabs Inc.). To compensate for sample with low RNA input, adaptors and primers were diluted 1:2 in nuclease-free water. Index sequences made of six-base combinations were then incorporated into the PCR products. Size selection of purified PCR products by QIAquick PCR purification kit (Qiagen, Germany) was performed by 6% polyacrylamide (PAGE) gel electrophoresis, selecting bands of 140 base pairs. Fragment sizes of selected libraries were assessed using capillary electrophoresis (CE) followed by 75 cycles of single-end sequencing and sequenced by the Illumina MiniSeq platform (Illumina) to produce raw FASTQ files. Briefly, the quality of the sequenced reads was assessed using the FastQC application (distributed by Babraham Institute, Cambridge, UK).

3.8.3 Screening of exosomal-microRNA via Nanostring

The multiplexed NanoString nCounter miRNA expression assay (Human v3 miRNA panel) was performed to profile 800 human miRNAs (NanoString Technologies, USA). The assay was conducted according to manufacturer's manual. A total exosome RNA input of 100ng was used as input material. For NanoString nCounter miRNA sample preparation, RNA input was mixed with pairs of capture and reporter probes tailored to each miRNA for hybridization, followed by removal of unligated material via enzymatic purification. The aligned and immobilized probe-bound miRNAs on a surface of nCounter Cartridge were quantified by scanning the fluorescent color-coded bar tags on the reporter probes with max-density scan (555 fields of view per sample). Probes for several housekeeping genes (HKG) such as ribosomal protein L10 (RPL10), beta-actin (ACTB), B2M, glyceraldehyde 3-phosphate dehydrogenase (GAPDH), and ribosomal protein L19 (RPL19) were incorporated in the Nanostring codesets and used for analysis along with positive and negative controls. Apart from that, HKG were used to evaluate cellular RNA contamination in biofluid samples.

3.9 Data analysis of exosomal-miRNAs profile

3.9.1 Data analysis of next generation sequencing via CLC Benchwork 8.0

The adapter sequence from raw NGS data were trimmed and trimmed data were filtered to remove sequences with read length shorter than 17 and more than 27 bases via CLC Genomics Workbench 8.0. The normalised trimmed read lengths were aligned to the Ensembl human database (Homo sapiens GRCh 37.57) and known miRBase-21 (*Homo sapiens*) database. The number of reads mapped to a miRNA was taken to represent the expression level of particular miRNA. The differential profiling of genome-wide miRNAs between each subpopulation relative to pMDA was then compared using Baggerley's t-test. The list of statistically significant differentially expressed miRNAs (fold change $\geqslant 2$, $p < 0.05$) relative to pMDA were generated.

3.9.2 Data analysis of Nanostring via nSolver 4.0

Quality of Nanostring data was assessed based on four default parameters, including imaging, binding density, positive control linearity and positive control limit of detection. Background correction was performed on detected miRNA from Nanostring nCounter by subtracting mean plus thrice the standard deviation (SD) of the eight negative controls as a cut-off point. For technical variations, normalisation was performed using geometric mean of top 100 expressed miRNAs as normalisation factor. Student t-test was conducted on differentially expressed miRNA. The list of statistically significant differentially expressed miRNAs (fold change $\geqslant 2$, $p < 0.05$) relative to pMDA were then generated.

3.9.3 Compilation of list of differentially regulated exosomal-miRNAs from next generation sequencing and Nanostring

The isolated exomiRNAs from each MDA subpopulation was profiled by two platforms which were NGS and Nanostring. Results acquired from NGS and Nanostring were filtered and processed prior to compilation of list of differentially regulated exomiRNAs for further analysis and identification of commonly dysregulated exomiRNAs (**Figure 3.2**).

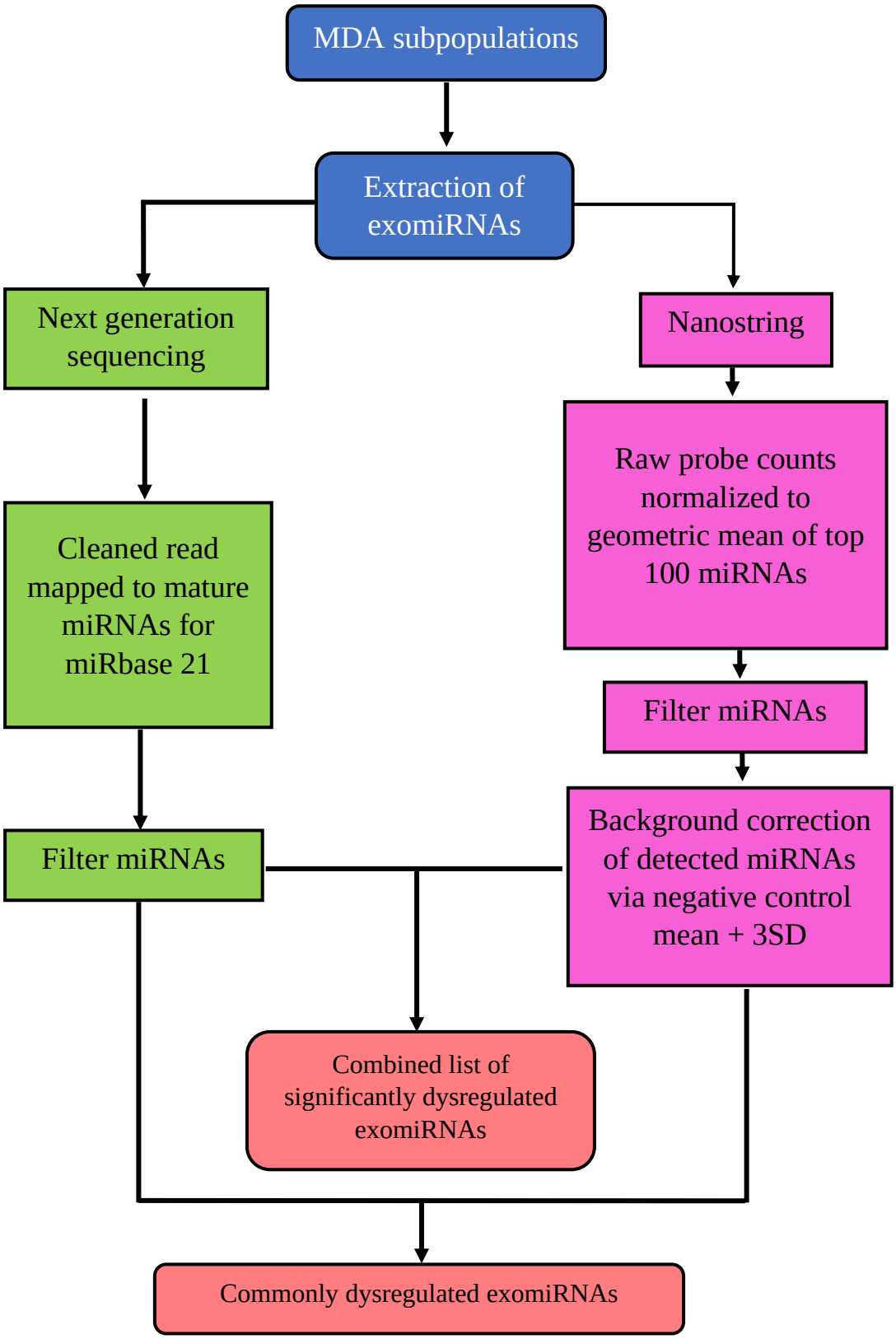


Figure 3.2: Overall study design and workflow of transcriptomic data analysis. Commonly dysregulated exomiRs detected in both NGS and Nanostring. MiRNAs which failed to pass the sample QC for respective panel were excluded from differential miRNA expression analysis.

3.9.4 Interaction networks between miRNAs-target genes and the functional analysis of target genes

MiRNA is known to targets more than one gene, and one gene is known to be targeted by more than one miRNA. Target genes of miRNAs were predicted using miRNet (http://www.mirnet.ca/), an online comprehensive tool integrated data from eleven different miRNA databases (TarBase, miRTar- Base, miRecords, miRanda, miR2Disease, HMDD, PhenomiR, SM2miR, PharmacomiR, EpimiR and starBase) (Y. Fan et al., 2016). MiRNet was used to visualise the complex underlying networks between the miRNA and their target genes. Enrichment pathway analysis of the predicted miRNAs targets were then performed using Kyoto Encyclopedia of Genes and Genomes (KEGG) where acquired p-values < 0.05 were regarded as statistically significant for the functional analysis.

3.10 Statistical analysis

Data were analysed and graphs were generated using PRISM v8.0 (Graph pad software) with analysed standard error of mean (SEM) displayed as error bar in the respective generated bar charts. Data acquired from RealTime-Glo™ MT Cell Viability and Annexin V/PI assays were analysed using student unpaired t-test. Remaining bioassays including CD24/CD44 and ALDH1A1 expression, migration assay, invasion assay, cell cycle analysis and mammosphere formation efficiency test were analysed using one-way analysis of variance (one-way ANOVA) followed by multiple comparisons with Tukey correction (comparison of mean differences between groups). All data were expressed as mean ± SEM of three independent biological replicates. The results were considered to be statistically significant at a probability level of p value < 0.05

Chapter 4

Result

4.1 Phenotypic characterisation of cisplatin-treated MDA-MB-231 subpopulations

4.1.1 Determination of IC_{50} value for cisplatin via MTT assay

MTT assay was conducted to evaluate the cytotoxicity of cisplatin based on the cellular metabolic activity of pMDA. The IC_{50} value was determined for selection of suitable time point and dosage of generating cisplatin-treated subpopulation for further bioassays. Cisplatin displayed its cytotoxic effect in dose dependent manner where the evaluated IC_{50} value of cisplatin were > 200 µM, 55.89 ± 2.12 µM and 36.23 ± 1.64 µM for 24, 48 and 72 hours respectively (**Figure 4.1**). The shortest time point where cells achieved stable cytotoxic effect was selected to acquire cisplatin-treated subpopulations.

The IC_{50} value of cisplatin at 24 hours exceeded the threshold of 200µM, which was beyond the self-adjusted optimised treatment range of cisplatin concentration for this study, in reference to the range used in previous breast cancer studies (Boo et al., 2016; Mirmalek et al., 2016).

Indeed, the acquired IC_{50} value of pMDA reduced drastically for > 3.5-fold at 48 hours as compared to 24 hours, but to a much lower extent (1.7-fold) at 72 hours as compared to 48 hours, showing smaller decrement of IC_{50} value of cisplatin from 48 hours to 72 hours, indicating 48 hours as the tested shortest time point to achieve stable cisplatin cytotoxic effect. Therefore, the IC_{50} value of cisplatin treatment at 48 hours (56µM) was selected to treat both pMDA and CSC-MDA subpopulation for comparison of the effect of this drug in the cells.

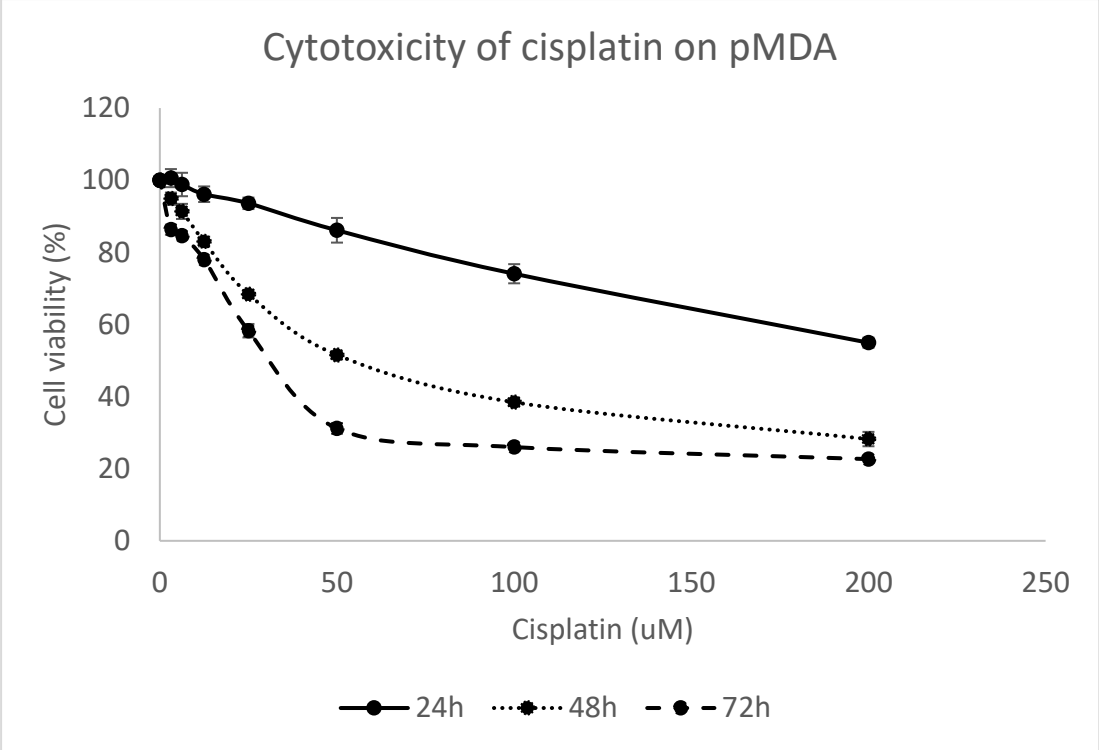


Figure 4.1: **Cell viability of pMDA after 24, 48 and 72 hours of cisplatin treatment.** The values are represented as means ± SEM for triplicate of independent experiments.

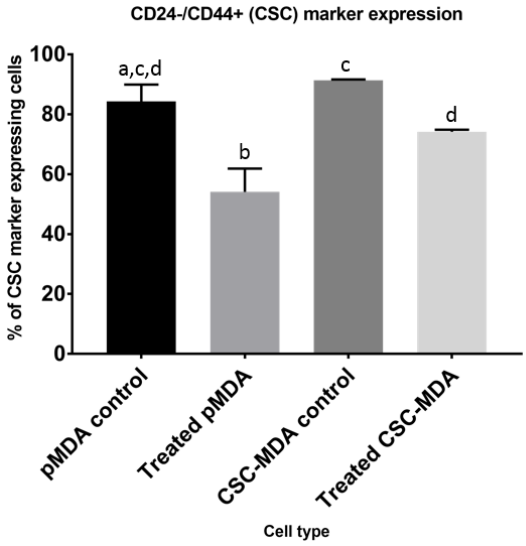
4.1.2 Expression of breast cancer stem cell surface markers ($CD24^-/CD44^+$) in cisplatin-treated subpopulations

Previous study showed that cisplatin treatment was able to induce cell differentiation therefore increased the expression of CD24 and reduced CSCs subpopulations (S. Y. Park et al., 2010; Prabhakaran et al., 2013). In this study, cisplatin treatment decreased the expression of CSCs markers on pMDA and CSC-MDA significantly compared to their respective controls, but to a lower extent in CSC-MDA (**Figure 4.2A**). Besides, cisplatin treatment has increased the expression of CD24 to a higher extent in pMDA as compared to CSC-MDA (**Figure 4.2B)**.

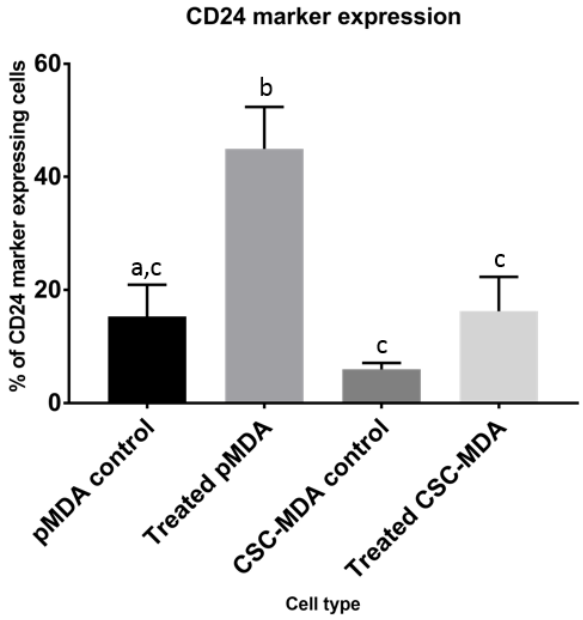
In treated CSC-MDA subpopulation, 74.15 ± 0.69% of CSC-like cells ($CD24^-/CD44^+$) were detected, which was significantly higher ($p = 0.0055$) than that of treated pMDA which consisted of 54.12 ± 7.78% of CSC-like cells. Other than that, CSC-MDA displayed high CD44 surface marker expression (red), low or absence of CD24 surface marker expression (green) (**Figure 4.2C**). Complementing results obtained from flow cytometry analysis,

immunofluorescent microscopy imaging showed that CD44 surface marker was almost equally expressed in treated pMDA and CSC-MDA while CD24 surface marker was more visible in treated pMDA. In fact, CD24 expression was significantly increased in treated pMDA (**Figure 4.2B**), which was observed as faint green signal indicated by white circle, as compared to pMDA while the increment of CD24 expression green signal in treated CSC-MDA relative to CSC-MDA was not observable. In other words, treated CSC-MDA that displayed relatively lower expression of CD24 marker and equally visible CD44 expression acquired higher CSCs feature as compared to treated pMDA. Therefore, in combination of the quantitative and qualitative result, CSCs subpopulation has retained higher percentage of CSC-like cells after cisplatin treatment as compared to its parental cells.

A


CD24-/CD44+ (CSC) marker expression
% of CSC marker expressing cells
100
80
60
40
20
0
a,c,d
b
c
d
pMDA control
Treated pMDA
CSC-MDA control
Treated CSC-MDA
Cell type

B


CD24 marker expression
% of CD24 marker expressing cells
60
40
20
0
a,c
b
c
c
pMDA control
Treated pMDA
CSC-MDA control
Treated CSC-MDA
Cell type

C

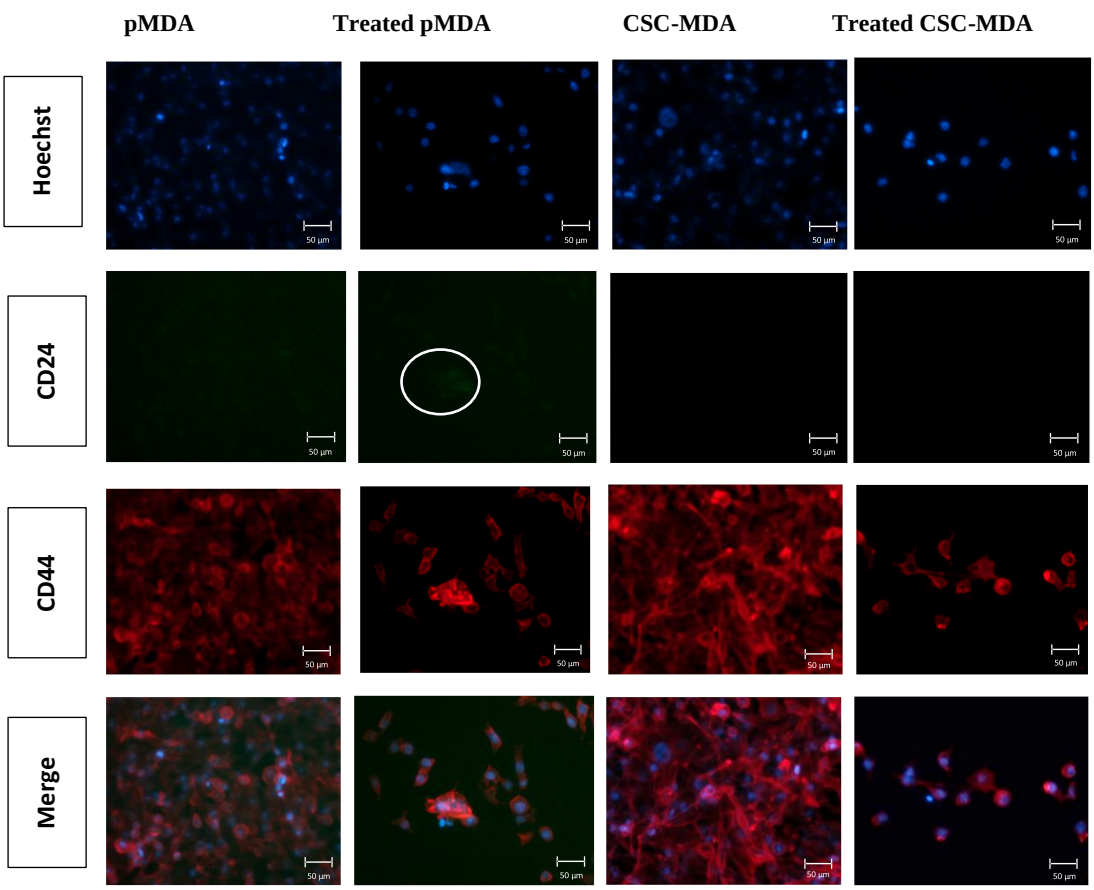


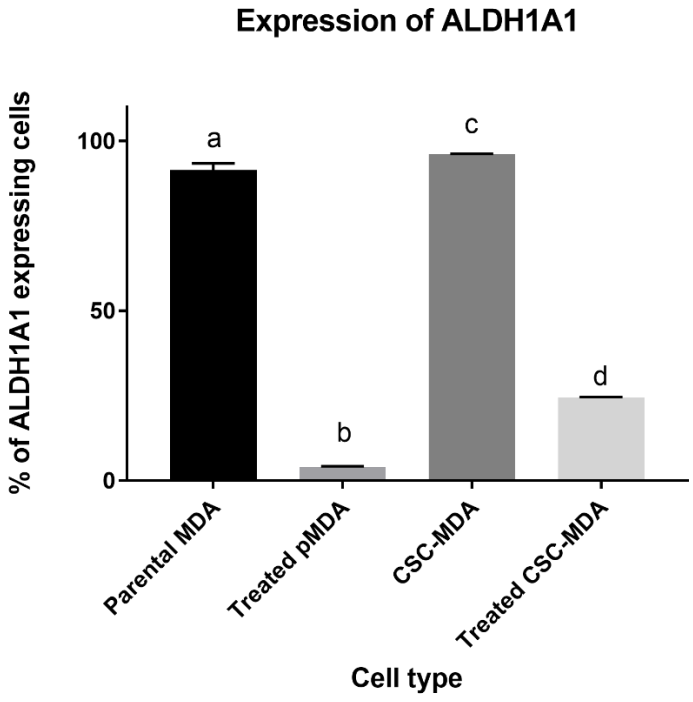
Figure 4.2: **Breast cancer stem cell surface markers detected using flow cytometry and fluorescent microscope**. (A) Expression of $CD24^{-}/CD44^{+}$ by control and treated pMDA and CSC-MDA, (B) expression of CD24+ on control and treated pMDA and CSC-MDA. Data were presented as means ± SEM for triplicate of independent experiments with different letters showing significantly difference where $p < 0.05$, (C) immunofluorescence imaging of control and treated pMDA and CSC-MDA at 200x magnification, stained by anti-CD24-FITC (green), anti-CD44-Alexa Fluor 647 (red) and Hoechst stain (blue) under the respective filters and a merged image of 3 filters where white circle under treated-pMDA indicates detected CD24-green signal.

4.1.3 Expression of intracellular breast cancer stem cell marker (ALDH1A1) in cisplatin-treated subpopulations

Other than detecting expression of CD24 and CD44 expression, flow cytometry analysis was also used to assess putative stem cell with high expression of another CSCs biomarker, ALDH1A1 (Vassalli, 2019). In this study, cisplatin treatment decreased the expression of ALDH1A1 on pMDA and CSC-MDA significantly compared to their respective controls, but to a lower extent in CSC-MDA (**Figure 4.3A**).

In treated CSC-MDA subpopulation, 24.37 ± 0.22% of cells were detected to express ALDH1A1, which was significantly higher ($p < 0.0001$) than that of treated pMDA that consisted of 4.04 ± 0.15% of ALDH1A1-expressing cells (**Figure 4.3A**). In accordance to results obtained from flow cytometry analysis, the immunofluorescent microscope imaging showed higher number of ALDH1A1-expressing cells in treated CSC-MDA than that of treated pMDA, which was visualised by higher intensity of the red signal (**Figure 4.3B**). With that, this set of data suggested that CSC-MDA expressed higher percentage of ALDH1A, which indicate higher detainment of CSC-like cells after cisplatin treatment as compared to its parental cells.

A



B

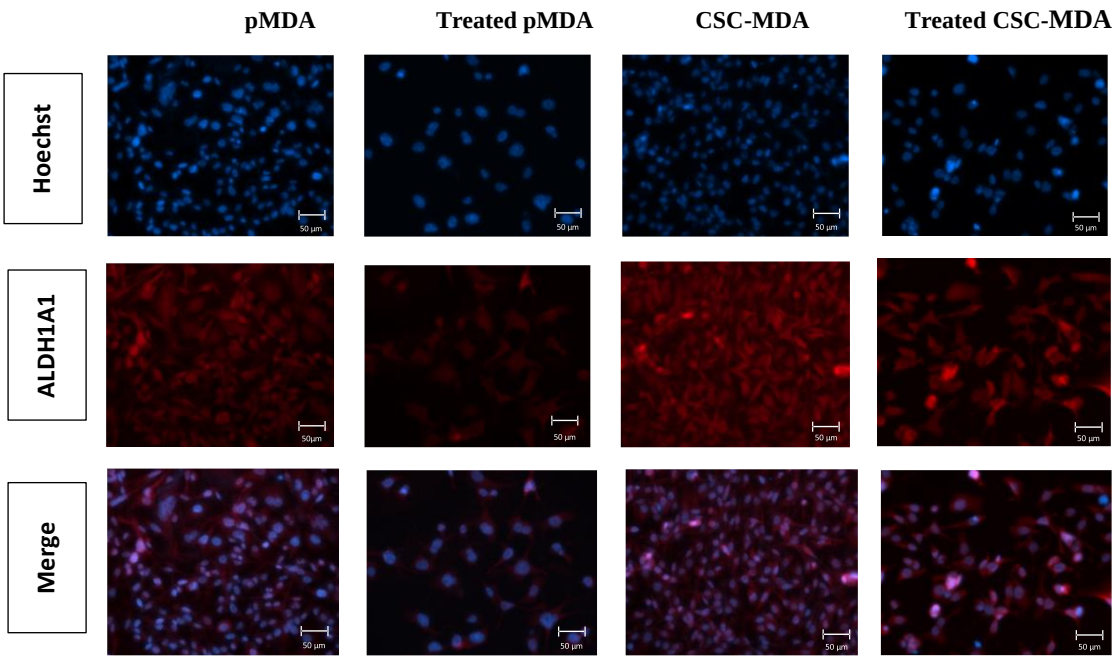


Figure 4.3: Intracellular breast cancer stem cell marker detected using flow cytometry immunophenotying assays. (A) Expression of ALDH1A1 on control and treated pMDA and CSC-MDA. Data were presented as means ± SEM for triplicate of independent experiments with different letters showing significantly difference where $p < 0.05$, (B) immunofluorescence imaging of control and treated pMDA and CSC-MDA at 200x magnification, stained by anti-ALDH1A1-APC (red) and Hoechst stain (blue) under the respective filters and a merged image of 2 filters.

4.2 Cytotoxic effect of cisplatin on MDA-MB-231 parental and breast cancer stem cell-like cells

4.2.1 Rate of migration and invasion of cisplatin-treated subpopulations

Breast CSCs are known to be highly metastatic that can lead to the growth of secondary tumour and cancer recurrence in breast cancer patients. In this study, invasion and migration assays were conducted to study the migratory ability and invasiveness of CSCs that is closely related to cancer metastasis.

Prior to cisplatin treatment, CSC-MDA showed significantly higher rate of migration by 19% ($p < 0.05$) and approximately 8% higher rate of invasion as compared to pMDA (**Figure 4.4**).

After cisplatin treatment was applied, rate of migration was suppressed significantly ($p < 0.05$) in treated CSC-MDA but insignificantly in treated pMDA as compared to their respective controls. However, although insignificant, treated CSC-MDA presented approximately 8% higher rate of migration as compared to treated pMDA (**Figure 4.4A**). On the other hand, rate of invasion was suppressed insignificantly in both treated subpopulations as compared to their respective controls. Although insignificant, treated CSC-MDA showed a trend of higher invasiveness than that of treated pMDA (**Figure 4.4B**).

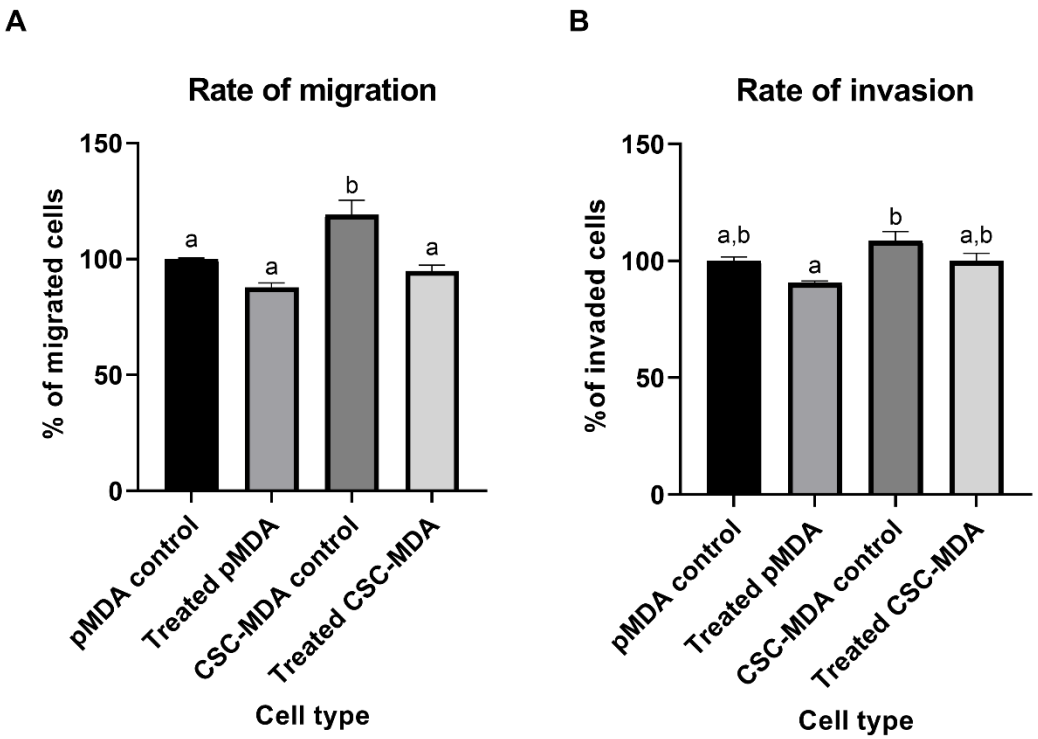


Figure 4.4: **Rate of migration and invasion of MDA subpopulations.** (A) Percentage of migrated cells after 24 hours and (B) invaded cells after 72 hours after cisplatin treatment. Data were presented as means ± SEM for triplicate of independent experiments with different letters showing significant difference where $p < 0.05$.

4.2.2 Induction of apoptotic event in cisplatin-treated subpopulations

Reduction of cell viability after cisplatin treatment was mainly due to the induction of apoptosis. With that, FITC Annexin V Apoptosis Detection Kit I was used to evaluate cisplatin-induced apoptosis event in pMDA and CSC-like cells.

Breast CSCs often demonstrate higher tolerance to chemotherapy resulting in lower event of apoptosis (Abdullah & Chow, 2013). In consistent to that, this study showed that cisplatin exerted different degree of apoptosis on pMDA and CSC-MDA. For instance, treated CSC-MDA displayed 46.97 ± 1.44% early apoptotic event, which was significantly higher ($p = 0.0001$) than that of treated pMDA that consisted of 4.45 ± 1.13% of early apoptotic event (**Figure 4.5**). However, 2.80 ± 1.76% of late apoptosis/necrosis event were detected in treated CSC-MDA subpopulation, which was significantly lower ($p = 0.0032$) than that of treated pMDA that displayed 48.64 ± 7.02% of late apoptosis/necrosis event (**Figure 4.5**). This finding

shows that treated CSC-MDA demonstrated significantly higher event of early apoptosis but significantly lower late apoptosis/necrosis as compared to treated pMDA.

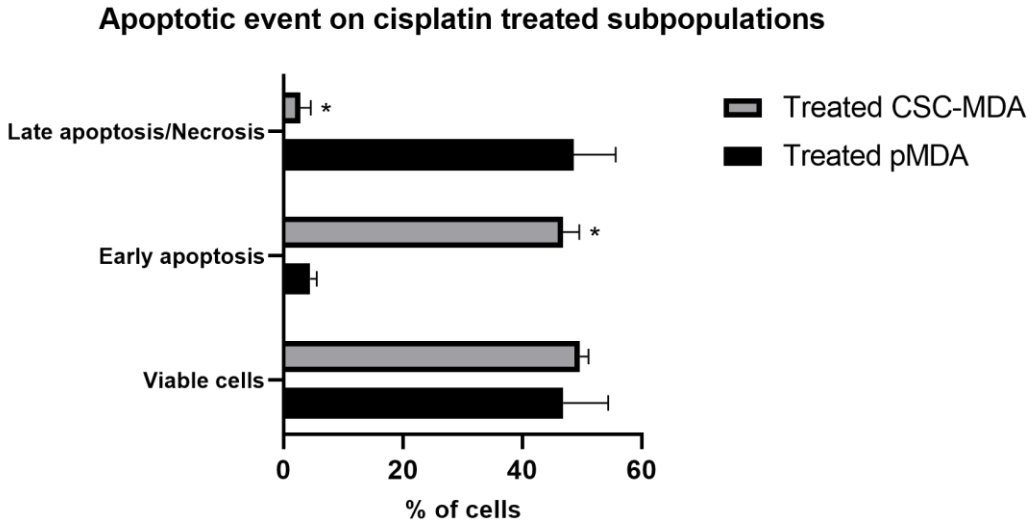


Figure 4.5: **Comparison of apoptotic events on pMDA and CSC-MDA after 48 hours of cisplatin treatment.** All data were expressed as means ± SEM for triplicate of independent experiments where * represents significant difference ($p<0.05$) as compared to treated pMDA.

4.2.3 Cell cycle analysis of cisplatin-treated subpopulations

One of the characteristics of breast cancer cell is uncontrollable cell division, which is modulated by cell cycle (Hanahan & Weinberg, 2000). With the aim to find out the effect of cisplatin on cell cycle distribution of pMDA and CSC-MDA, DNA content in cells were evaluated using BD Cycletest plus DNA kit where result was acquired by flow cytometry.

First of all, effect of cisplatin exclusively on G0/G1, S and G2/M phases for treated subpopulations were evaluated by excluding sub G0/G1 phase (**Figure 4.6A**). This exclusive comparison enabled precise evaluation of cisplatin treatment's interference on these three phases.

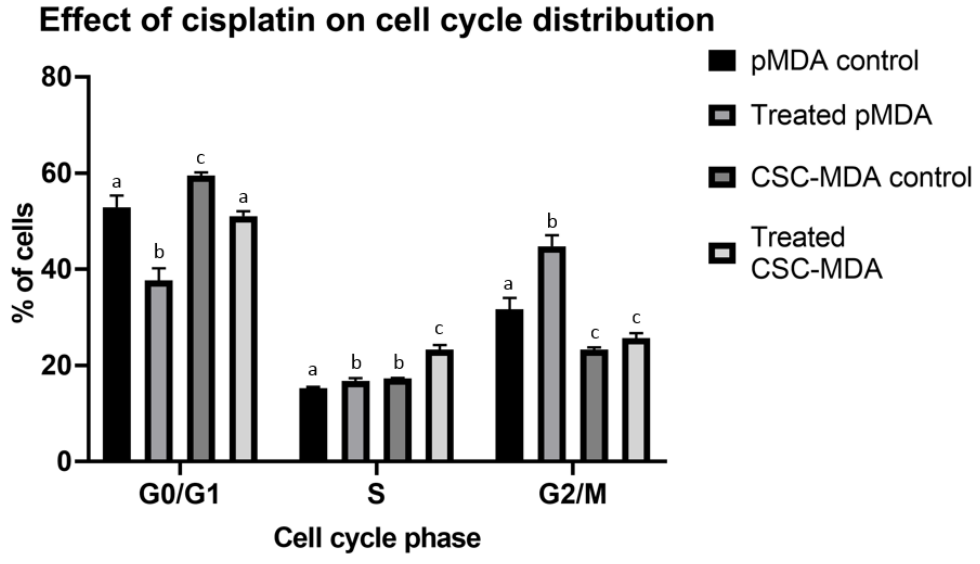
As compared to their respective controls, cisplatin triggered higher and more significant cell increment in S phase in treated CSC-MDA (increased by 5.98%; $p < 0.0001$) than that of treated

pMDA (increased by 1.53%; $p < 0.05$), whereas solely treated pMDA displayed significant ($p < 0.0001$) cell increment in G2/M phase by 13.12% as compared to pMDA (**Figure 4.6A**).

Besides, sub G0/G1 content of treated CSC-MDA were significantly increased by 26.83% ($p < 0.0001$) as compared to CSC-MDA control, which the increment was 7.2% lower than treated pMDA that increased by 34.03% ($p < 0.0001$) as compared to pMDA control (**Figure 4.6B**).

These data showed that cisplatin induced a small portion of treated CSC-MDA arrested at S phase while significant percentage of treated pMDA was arrested at G2/M phase. Furthermore, cisplatin induced increment of sub G0/G1 event in both treated subpopulations but to a higher extent in treated pMDA as compared to treated CSC-MDA.

A



B

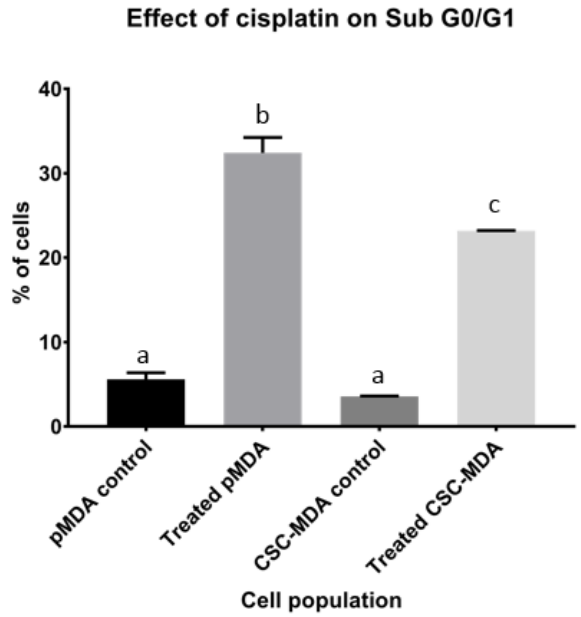


Figure 4.6: Flow cytometric analysis of cell cycle distribution after cisplatin treatment. (A) Effect of cisplatin treatment on cell cycle distribution of pMDA and CSC-MDA, evaluated exclusively on G0/G1 phase, S phase and G2/M phase and (B) solely on sub G0/G1 phase. Data were presented as means ± SEM for triplicate of independent experiments with different letters showing significant difference where $p < 0.05$.

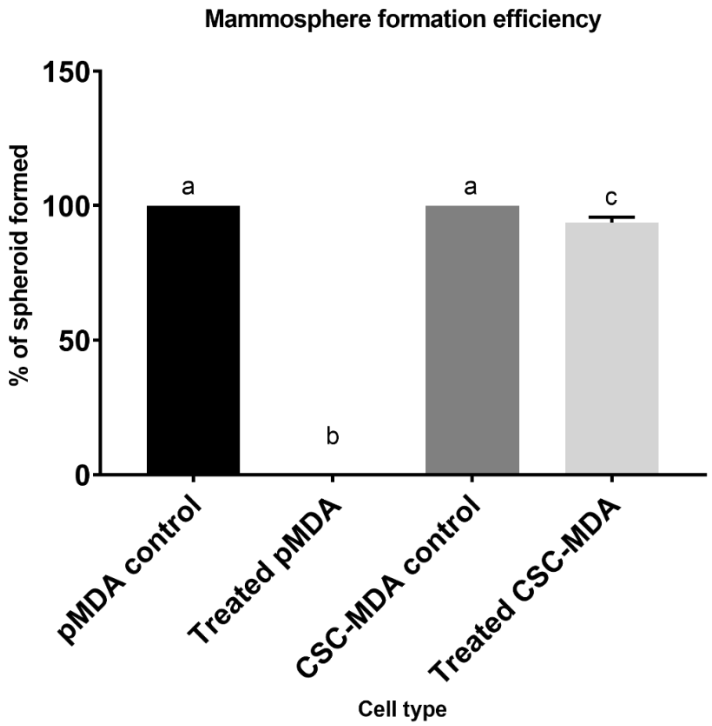
4.2.4 Self-renewability of cisplatin-treated subpopulations

Self-renewability is known as one of the traits acquired by breast CSCs that enable them to retain their stemness at the same time to propagate cancer cells that are capable to undergo cell differentiation, eventually generate secondary tumour in the patient (Lawson et al., 2009). Mammosphere formation efficiency test was used to investigate the self-renewability of each subpopulation.

Although both pMDA and CSC-MDA controls possessed similarly high self-renewability, only treated CSC-MDA retained self-renewability despite significant reduction after cisplatin treatment. This was observed by 90.97 ± 1.84% of generated mammosphere in treated CSC-MDA, which was significantly higher ($p < 0.0001$) than that of treated pMDA, which failed to generate mammosphere (**Figure 4.7A**). Interestingly, although pMDA and CSC-MDA controls generated 100% of mammosphere before treatment, the size of generated mammosphere

between these cells were different. Larger but less compact mammospheres were observed in pMDA group with an average of 734.6 ± 36.1 µm while CSC-MDA generated smaller but compact mammosphere that average to about 416.5 ± 27.3 µm in size after 14 days in culture. In contrast to the morphology of their controls, treated CSC-MDA then generated larger mammosphere of estimated size of 68.4 ± 1.8 µm whereas treated pMDA failed to generate mammosphere (**Figure 4.7B**). These data showed that pMDA and CSC-MDA acquired high self-renewability while CSC-MDA displayed lower proliferation rate as compared to pMDA before treatment. On the other hand, cisplatin reduced both self-renewability and proliferation rate of CSC-MDA to a lower extent as compared to pMDA that completely lost its self-renewal and proliferation abilities after cisplatin treatment.

A



B

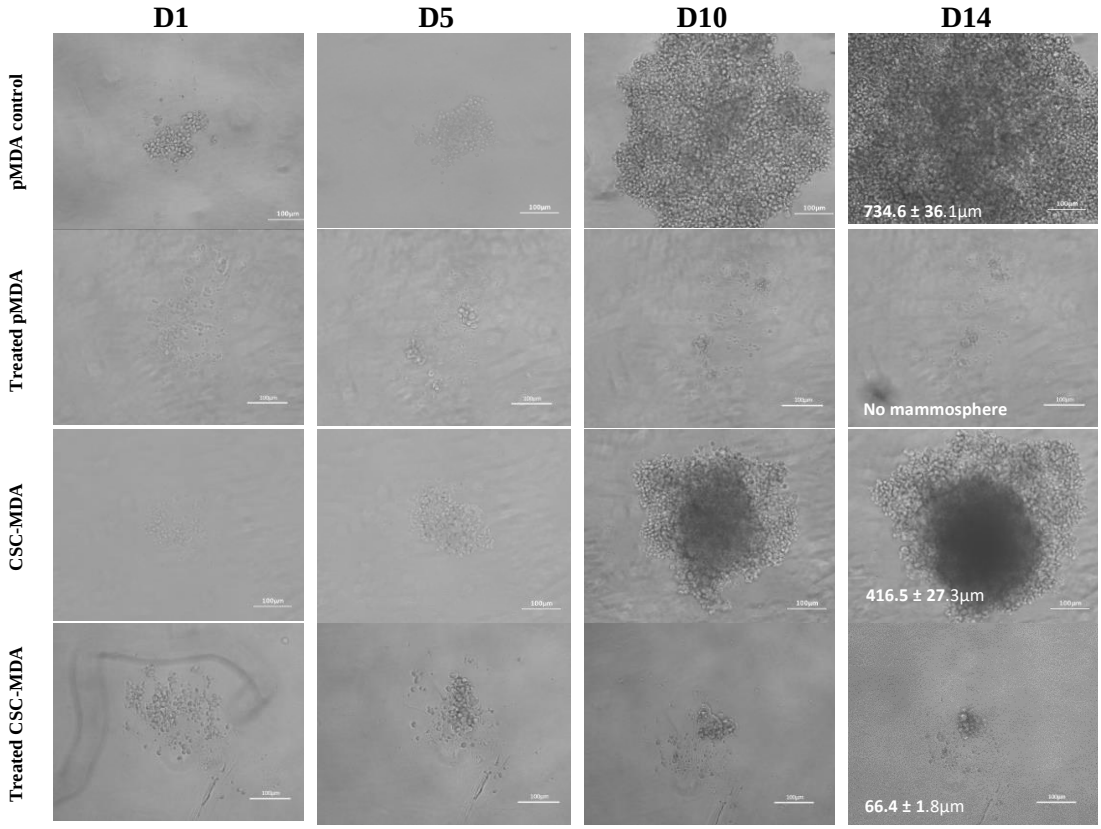


Figure 4.7: Percentage and sizes of generated mammosphere in each subpopulation. (A) Number of generated mammosphere was assessed after 14 days, (B) images of generated mammosphere at 10X magnification on day 14. Data were presented as means ± SEM for triplicate of independent experiments with different letters showing significant difference where $p < 0.05$.

4.3 Effect of cisplatin on cellular gene expression of MDA-MB-231 breast cancer cells

4.3.1 Profiling of cancer stem cell-correlated gene expression in control and cisplatin-treated pMDA vs CSC-MDA

Quantitative polymerase chain reactions (qPCR) is known as the gold standard for quantifying gene expression. Therefore, RT^2 profiler PCR array, which is a qPCR-based method with the features of mini-array, was used to study cellular gene expression, particularly on functionally correlated genes in CSCs, before and after cisplatin treatment to decipher the alteration of gene profiles in pMDA and CSC-MDA subpopulations.

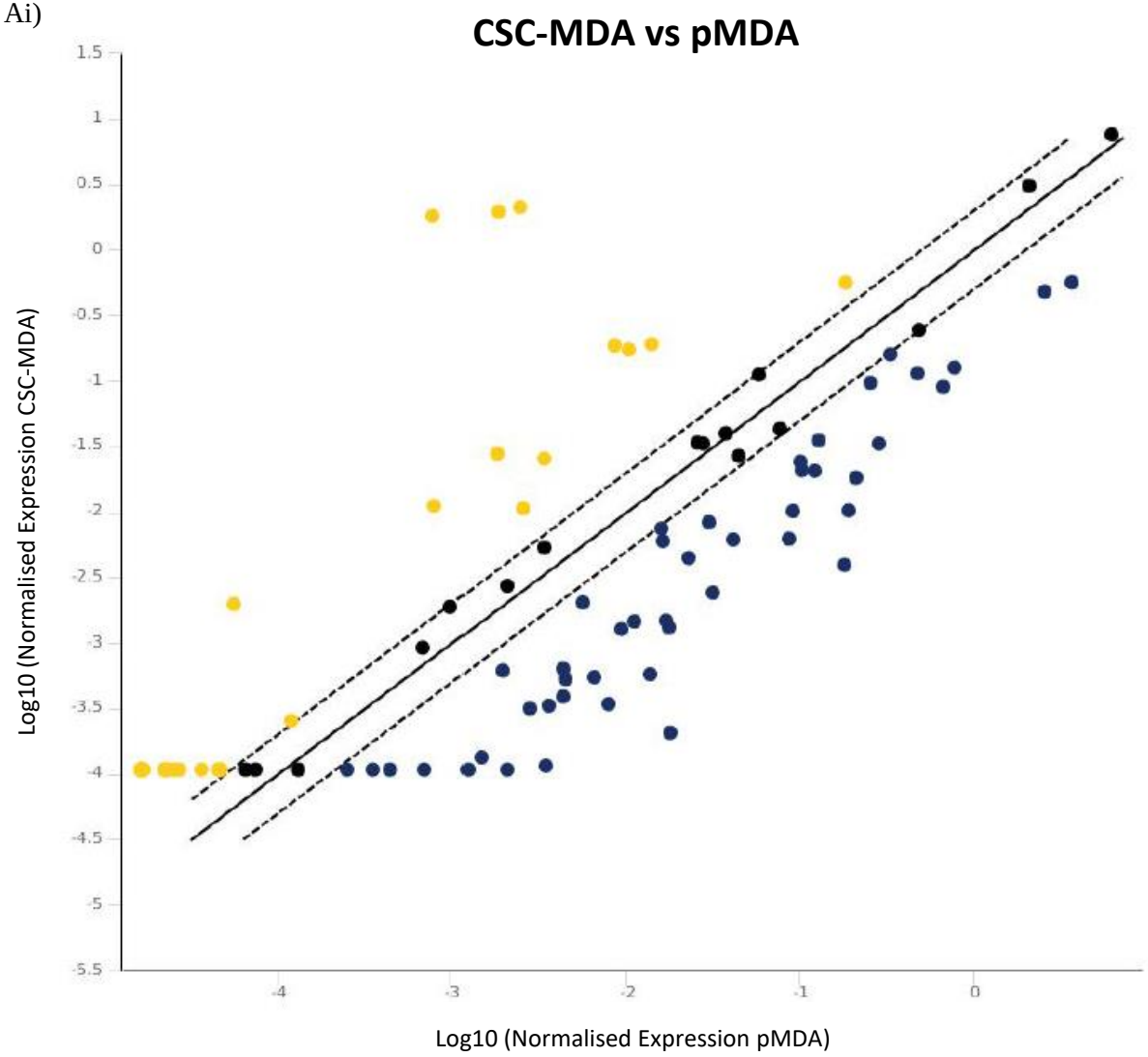
Scatter plots were used to compare the expression of CSC-correlated genes between control subpopulations (**Figure 4.8A**), cisplatin-treated subpopulations (**Figure 4.8A**), pMDA subpopulations (**Figure 4.8B**) and CSC-MDA subpopulations (**Figure 4.8B**).

Higher number of gene were upregulated in CSC-MDA with fewer genes being downregulated in CSC-MDA control as compared to pMDA control (**Figure 4.8A**). Furthermore, more genes were upregulated in treated CSC-MDA as compared to treated pMDA **Figure 4.8A**).

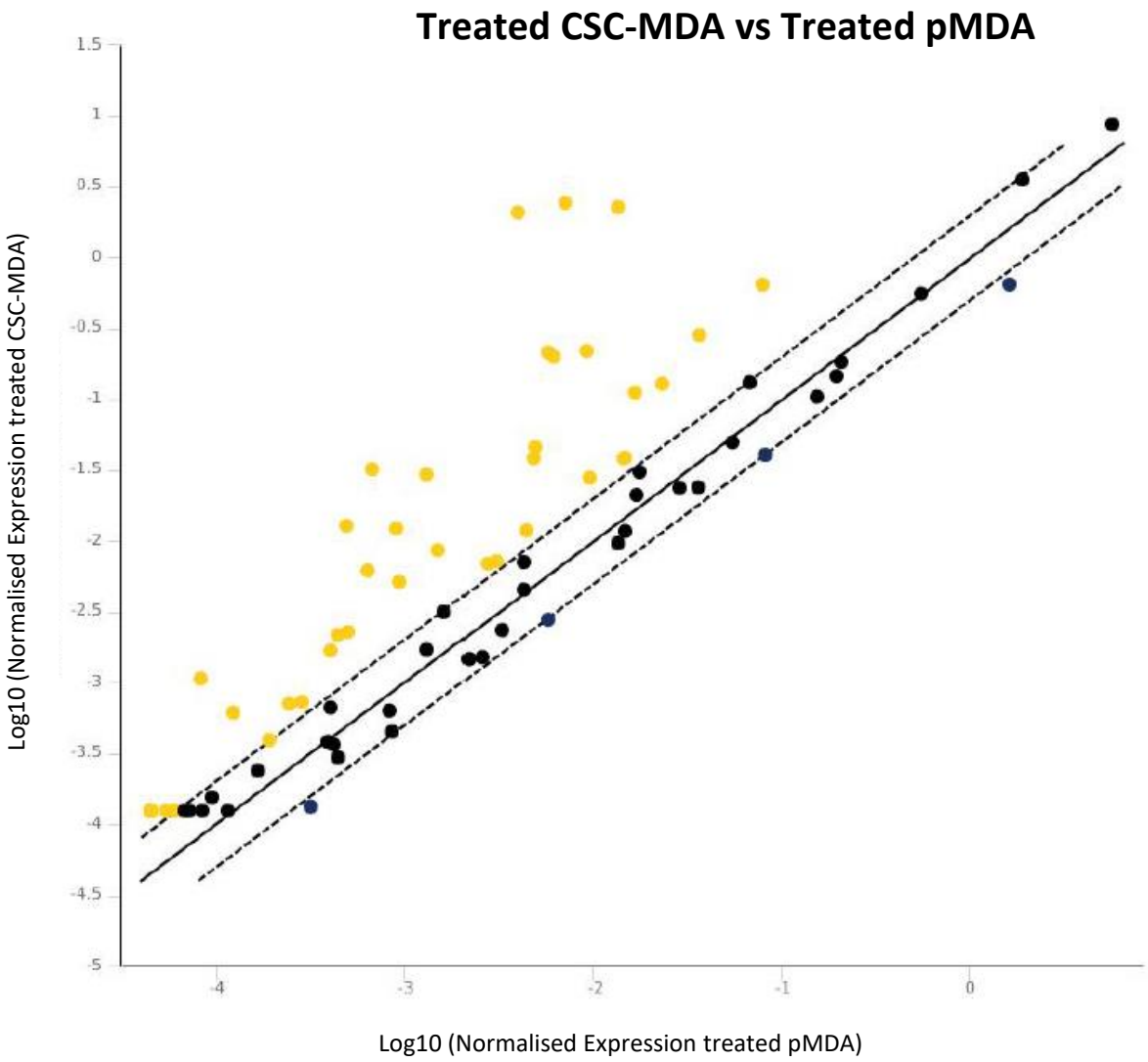
Not only that, more genes were found significantly downregulated for at least 2-fold in both treated subpopulations but to a higher extent in pMDA as compared to CSC-MDA (**Figure 4.8B**).

Together, these results indicated that CSC-correlated genes were more enriched in control and treated CSC-MDA as compared to pMDA subpopulation.

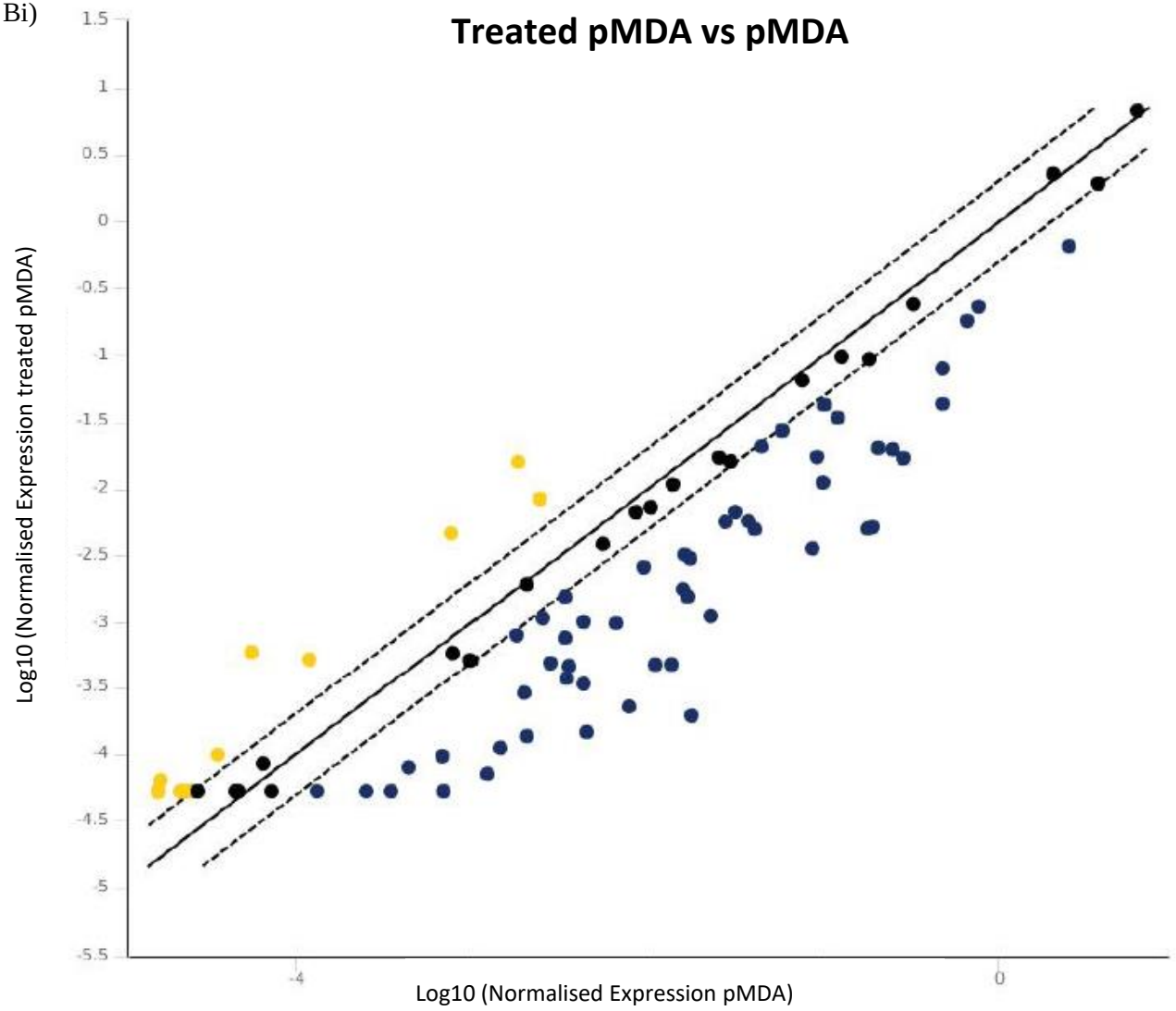
Ai)


CSC-MDA vs pMDA
Log10 (Normalised Expression CSC-MDA)
Log10 (Normalised Expression pMDA)
1.5
1
0.5
0
-0.5
-1
-1.5
-2
-2.5
-3
-3.5
-4
-4.5
-5
-5.5
-4
-3
-2
-1
0

Aii)


Treated CSC-MDA vs Treated pMDA
Log10 (Normalised Expression treated CSC-MDA)
Log10 (Normalised Expression treated pMDA)
1.5
1
0.5
0
-0.5
-1
-1.5
-2
-2.5
-3
-3.5
-4
-4.5
-5
-4
-3
-2
-1
0

Bi)


Treated pMDA vs pMDA
Log10 (Normalised Expression treated pMDA)
Log10 (Normalised Expression pMDA)
1.5
1
0.5
0
-0.5
-1
-1.5
-2
-2.5
-3
-3.5
-4
-4.5
-5
-5.5
-4
0

Bii)

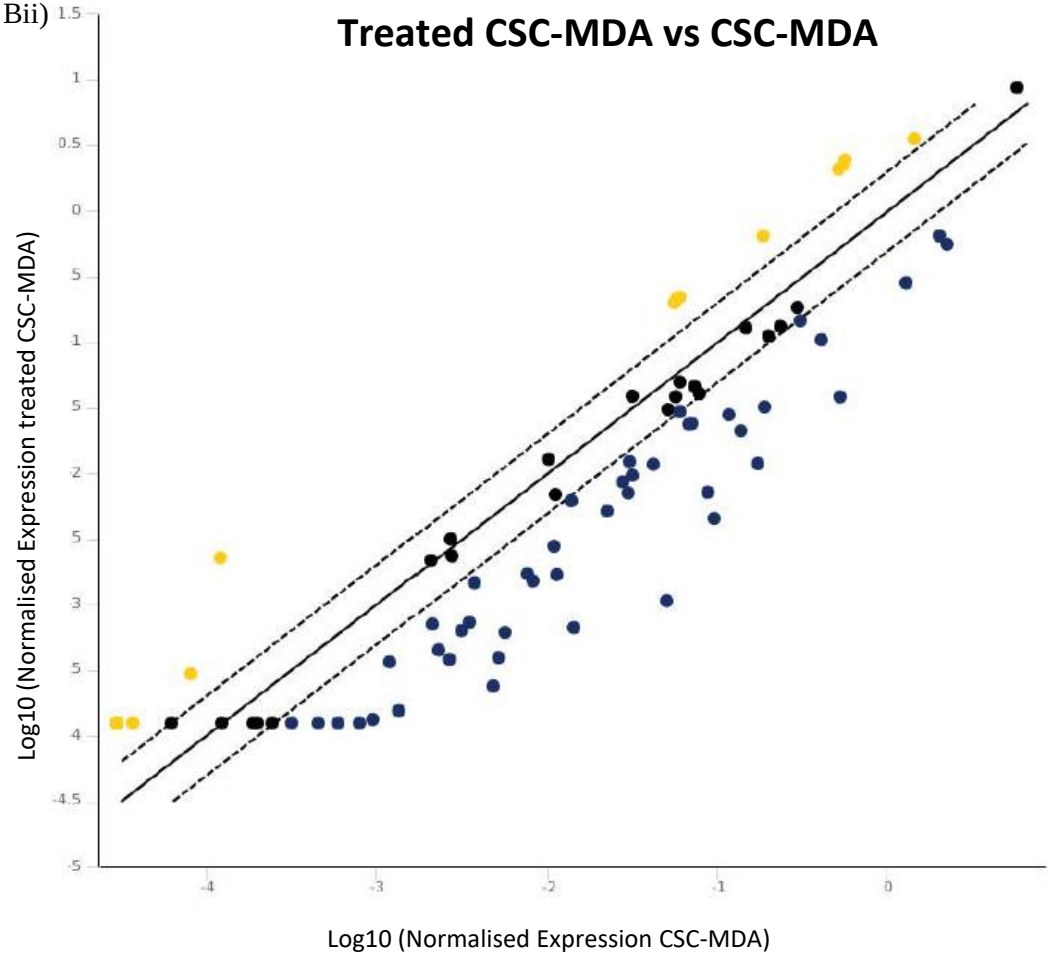


Figure 4.8: Scattered plots of CSC-related gene expression. (A) CSC-related gene expression profiles (i) before and (ii) after treatment in CSC-MDA subpopulation as compared to pMDA subpopulation. (B) CSC-related gene expression profiles of (i) treated pMDA and (ii) treated CSC-MDA as compared to their respective controls. Upregulated, downregulated and unchanged genes represented by yellow, blue and black dots respectively.

4.3.2 Dysregulated cancer stem cell-correlated genes and corresponding signalling pathway induced by cisplatin treatment

Based on the determined dysregulated gene profiles in respective subpopulations, enriched signalling pathways by these dysregulated genes were annotated via PANTHER overrepresentation test (**Figure 4.10**).

First of all, the acquired genotypic profile in CSC-MDA showed 17 significantly dysregulated genes ($\geqslant$2-fold, $p<0.05$), comprised of 12 upregulated genes and 5 downregulated genes as compared to pMDA. Among these genes, KIT ligand (KITLG) was the most upregulated gene while Krueppel-like factor 17 (KLF17) was the most downregulated gene (**Appendix 3-Table 3**). However, no significant pathway was annotated based on the dysregulated genes in CSC-MDA as compared to pMDA (result not shown).

Next, genotypic profiles of both cisplatin-treated subpopulations were compared to investigate the differently dysregulated CSC-correlated genes and their associated pathways induced by cisplatin in respective subpopulations. Data showed 34 significantly dysregulated genes by at least 2-fold, comprised of 33 upregulated genes with only 1 downregulated gene in treated CSC-MDA as compared to treated pMDA (**Appendix 3-Table 4**). Annotation analysis showed that four of these dysregulated genes were correlated with 'JAK/STAT' and 'Hh' signalling pathways (**Figure 4.10A**).

Cisplatin-induced significantly dysregulated genes ($\geqslant$2-fold, $p<0.05$) in pMDA and CSC-MDA relative to their respective controls were compared to identify the commonly and distinctively dysregulated gene profiles between pMDA and CSC-MDA (**Figure 4.9**). There were 33 commonly dysregulated genes between pMDA and CSC-MDA after cisplatin treatment where up to 32 genes were downregulated with only 1 upregulated gene detected (**Figure 4.9**). However, the only upregulated gene (DLL4) displayed a negligible difference of only 0.1-fold between the increment

in CSC-MDA (2.85-fold) and pMDA (2.74-fold) as compared to their respective controls (**Appendix 3-Table 1 & 2**). A number of these commonly dysregulated genes were significantly annotated to seven signalling pathways (**Figure 4.10B**). Among these pathways, more than 10% of these genes were involved in 'platelet-derived growth factor (PDGF)', 'Notch' and 'angiogenesis' pathways, which are correlated with breast cancer progression. Besides, there were 4 significantly dysregulated genes solely in pMDA subpopulation after cisplatin treatment, comprised of 1 upregulated gene and 3 downregulated genes (**Figure 4.9**). There was no significant pathway annotated based on these dysregulated genes in treated pMDA (result not shown). On the other hand, there were 37 significantly dysregulated genes solely in CSC-MDA subpopulation after cisplatin treatment, comprised of 21 upregulated genes and 16 downregulated genes (**Figure 4.9**). Eight of these exclusively dysregulated genes in treated CSC-MDA were significantly annotated to three signalling pathways, including 'Hh', 'cholecystokinin receptor (CCKR)' and 'angiogenesis' pathways (**Figure 4.10C**).

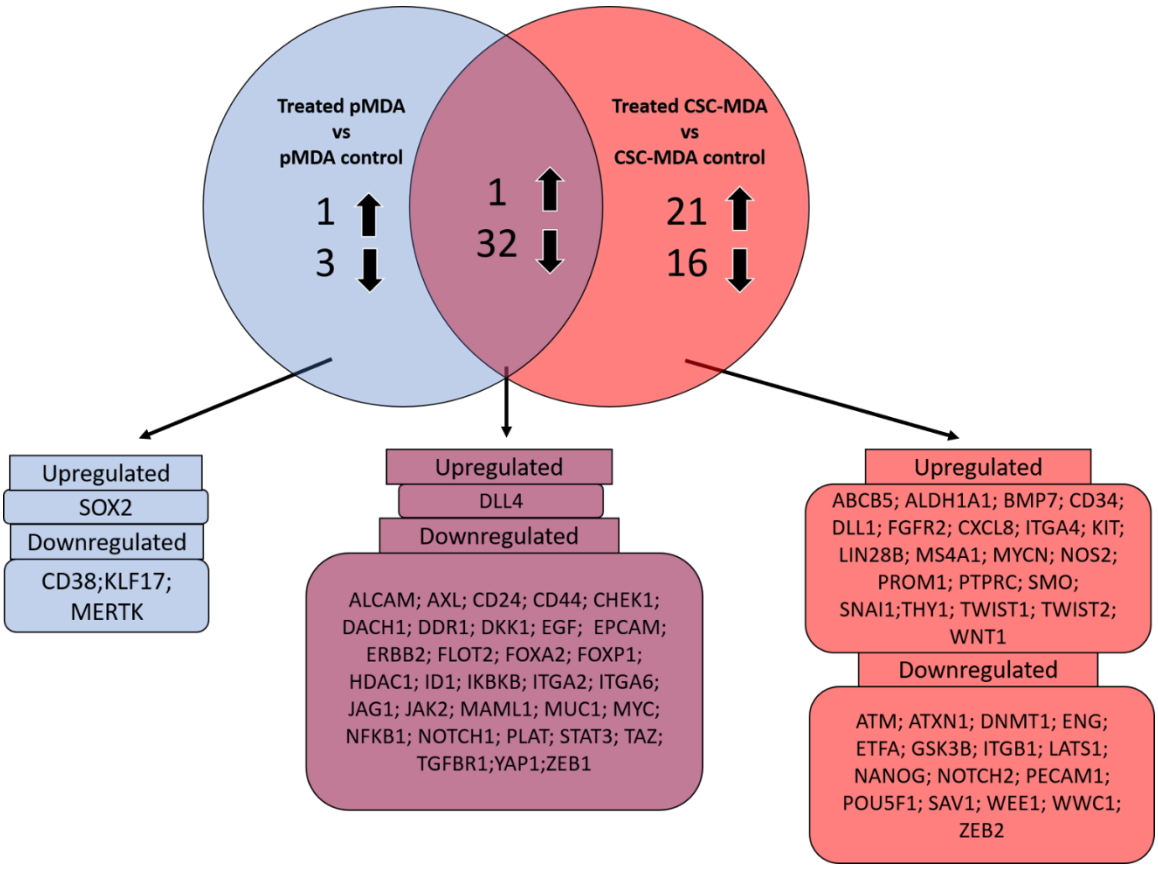
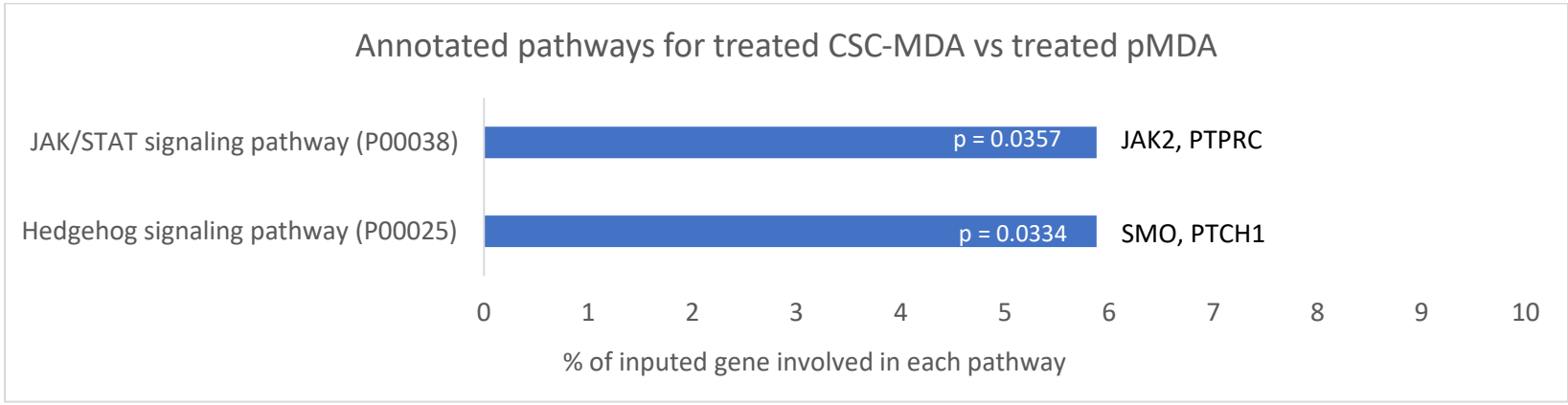
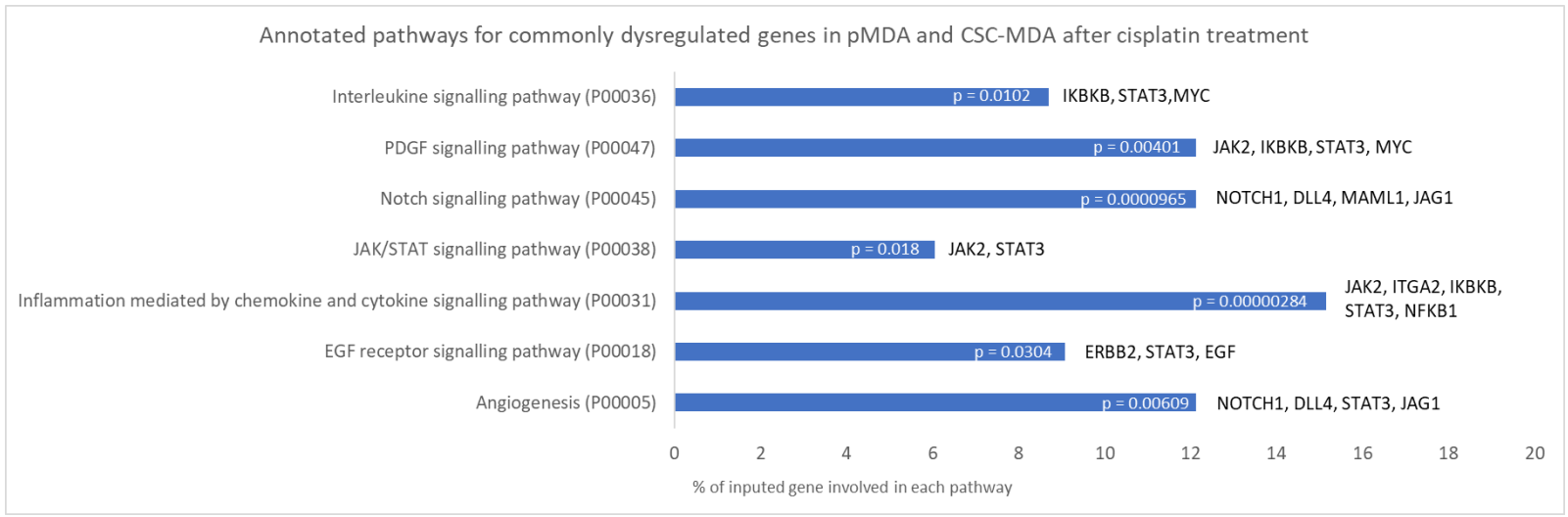


Figure 4.9: Venn diagram analysis for cisplatin-induced dysregulation of genes in pMDA and CSC-MDA. Number of significantly dysregulated CSC-correlated genes (≥2-fold, $p<0.05$) in pMDA and CSC-MDA subpopulation after cisplatin treatment, with uniquely and commonly expressed genes in treated pMDA vs pMDA control and treated CSC-MDA vs CSC-MDA control.

A


Annotated pathways for treated CSC-MDA vs treated pMDA
JAK/STAT signaling pathway (P00038)
p = 0.0357
JAK2, PTPRC
Hedgehog signaling pathway (P00025)
p = 0.0334
SMO, PTCH1
0
1
2
3
4
5
6
7
8
9
10
% of inputed gene involved in each pathway

B


Annotated pathways for commonly dysregulated genes in pMDA and CSC-MDA after cisplatin treatment
Interleukine signalling pathway (P00036)
p = 0.0102
IKBKB, STAT3,MYC
PDGF signalling pathway (P00047)
p = 0.00401
JAK2, IKBKB, STAT3, MYC
Notch signalling pathway (P00045)
p = 0.0000965
NOTCH1, DLL4, MAML1, JAG1
JAK/STAT signalling pathway (P00038)
p = 0.018
JAK2, STAT3
Inflammation mediated by chemokine and cytokine signalling pathway (P00031)
p = 0.00000284
JAK2, ITGA2, IKBKB, STAT3, NFKB1
EGF receptor signalling pathway (P00018)
p = 0.0304
ERBB2, STAT3, EGF
Angiogenesis (P00005)
p = 0.00609
NOTCH1, DLL4, STAT3, JAG1
0
2
4
6
8
10
12
14
16
18
20
% of inputed gene involved in each pathway

C

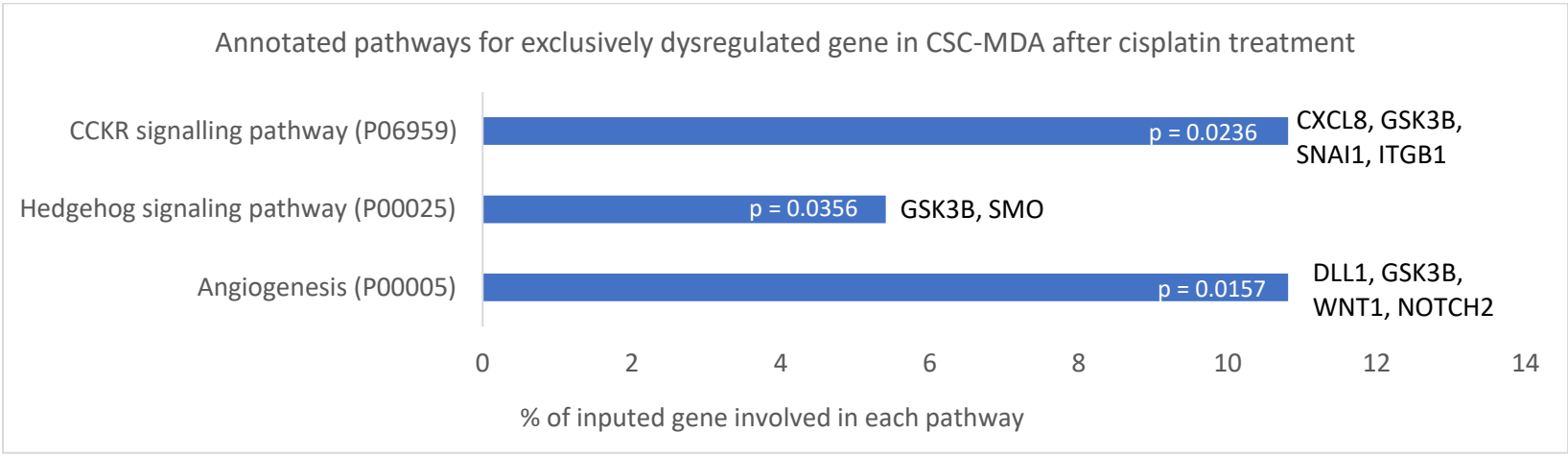


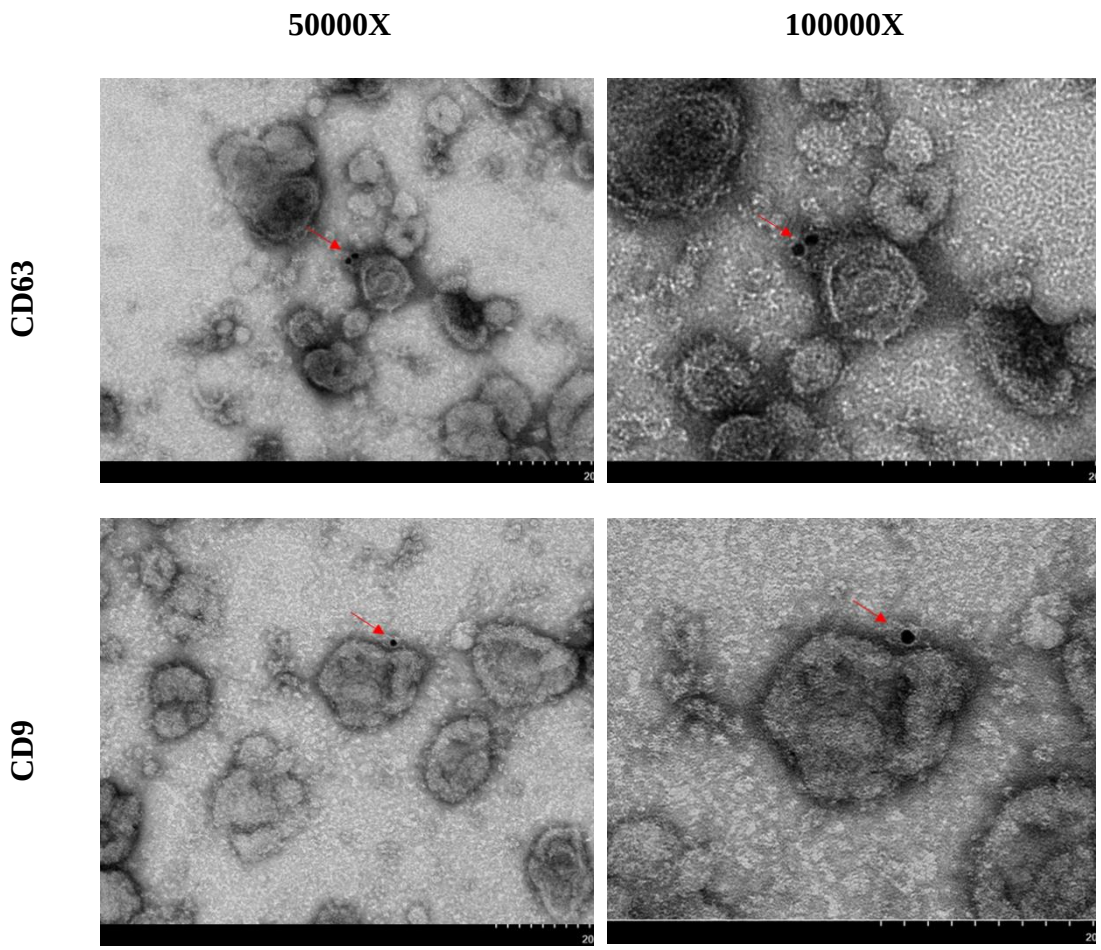
Figure 4.10: PANTHER-pathway analysis. (A) Annotated signalling pathways correlated to significantly dysregulated CSC-correlated genes (⩾2-fold, $p<0.05$) in treated CSC-MDA as compared to treated pMDA, (B) commonly dysregulated CSC-correlated genes (⩾2-fold, $p<0.05$) in pMDA and CSC-MDA after cisplatin treatment, and (C) exclusively dysregulated CSC-correlated genes (⩾2-fold, $p<0.05$) in CSC-MDA after cisplatin treatment. The corresponding adjusted Fisher's exact p values ($p < 0.05$) were presented for each pathway.

4.4 Exosome characterisation

4.4.1 Characterisation of MDA-MB-231 breast cancer secreted exosome via transmission electron microscope (TEM) and Zeta nano sizer

TEM and Zeta nano sizer were conducted to verify presence of exosome by assessing the expression of exosomal biomarkers (CD63 and CD9) and particle size (ranging from 10-200nm) of the isolated vesicles prior to miRNAs extraction. Both TEM and Zeta nano sizer analyses (**Figure 4.11**) showed the particle size of secreted MDA exosomes to be in the range 100–200 nm (mean size: 160.8 ± 39.81 nm). Furthermore, presence of exosomes were additionally confirmed by the exosome biomarkers CD63 and CD9, which were conjugate to gold particles (10 nm) as indicated by red arrow (**Figure 4.11A**).

A



B

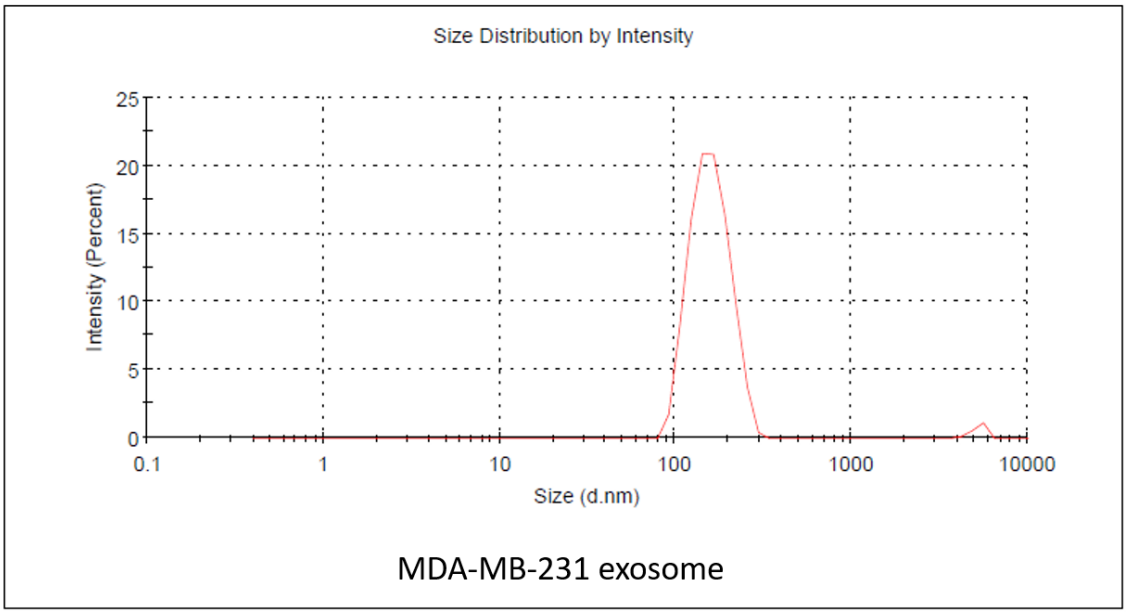


Figure 4.11. Exosome characterisation. (A) MDA exosomes were identified by transmission electron microscopy through biomarkers CD63 and CD9-gold particles (10 nm) as indicated by red arrow (left × 50,000 magnification; right × 100,000 magnification). **(B)** DLS of three average runs for MDA exosomes. The size distribution is depicted as the intensity of detection in function of the particle size displayed over a logarithmic scale.

4.5 Transcriptomic profiling of exosomal-miRNAs in MDA-MB-231 breast cancer cells

4.5.1 Comparison of exosomal-miRNAs profiles generated by NGS and Nanostring

All acquired NGS result for the triplicates of four subpopulations passed the quality control (QC) and were of high quality as indicated by FastQC. Based on the results obtained from NGS, a total of 10.28 million raw reads were generated for all the samples, yielding an average 7.978313 million clean reads. Besides, clean reads from four MDA subpopulations displayed a mapable reads of miRNAs in a range of 131 – 191 miRNAs (**Table 4.1**). On the other hand, quality of Nanostring data was assessed based on four default parameters, including imaging, binding density, positive control linearity and positive control limit of detection. Triplicate of all four subpopulations passed the QC assessment. The mean plus thrice the SD of the eight negative controls for each sample was subtracted from each miRNA count in that sample before proceeding to downstream analysis, resulting in detection of 206 – 231 miRNAs expressed above threshold across four subpopulations (**Table 4.1**).

As NGS profiled miRNA with CE precision while Nanostring is of digital precision, concordance and shared detection of miRNAs between these platforms were investigated. Based on result obtained, 30-35% of NGS-detected miRNAs was also detected in Nanostring for each subpopulation (**Table 4.1**). In addition, a similar overlap analysis was performed to investigate the top 25% of detected miRNAs from both platforms and the overlap were reduced to 15-21%. However, when comparing the highly expressed miRNAs (expression above 100 reads in NGS), between 50 – 100 % of miRNAs detected in NGS were expressed above threshold in Nanostring (**Table 4.1**). One point of note is that NanoString included only 798 miRNAs in the pre-designed chip whereas NGS obviously could detect any number of miRNAs present in the samples. Therefore, further analysis was performed to remove the miRNAs detected by NGS but not included in the NanoString chip, resulted in higher overlap between the panels (**Appendix 4**).

Table 4.1: Shared detection of exosomal-miRNAs in NGS and Nanostring

		pMDA	Treated pMDA	CSC-MDA	Treated CSC-MDA
Number of detected miRNAs	**NGS**	**170**	**191**	**148**	**131**
	Nanostring	**231**	**206**	**208**	**211**
Percentage of overlapped miRNAs* (%)		**32.94**	**30.37**	**31.76**	**34.35**
Percentage of overlapped top 25% miRNAs* (%)		**20.9**	**21.28**	**15.79**	**18.75**
Percentage of overlapped for highly expressed miRNAs (>100 counts) in NGS* (%)		**50**	**69.23**	**85.71**	**100**

* Percentage calculated based on overlapped detected miRNAs in NGS and Nanostring (expressed ⩾ 10 counts) as compared to total number of miRNAs detected in NGS

4.5.2 Differential expression of exosomal-miRNAs in MDA-MB-231 breast cancer cells

To maximise the investigation of distinct exomiRNAs profiles in CSC-like subpopulation and treated subpopulations relative to their parental control (pMDA), differential studies were performed on data acquired from both NGS and Nanostring. The total number of significantly dysregulated exomiRNAs detected by both panels for treated pMDA was lower than that of CSC-like subpopulations. Moreover, lower number of exomiRNAs were downregulated in treated pMDA while the opposite was observed in both control and treated CSC-like subpopulations, displayed by drastic increased number of downregulated exomiRNAs (**Table 4.2**). The most dysregulated exomiRNAs in CSC-like subpopulation, treated subpopulations as compared to pMDA and treated CSC-like subpopulation as compared to CSC-like subpopulation were listed in **Table 4.2**.

Table 4.2: List of up- and down-regulated exosomal-miRNAs (≥2-fold, p<0.05) detected from Nanostring and NGS:

A) Cisplatin-treated pMDA as compared to pMDA

Upregulated ExomiRs	Fold change	Downregulated ExomiRs	Fold change
hsa-miR-379-5p	16.63	hsa-miR-99a-5p	-7.16
hsa-miR-584-5p	14.29	hsa-miR-550a-5p	-4.74
hsa-miR-30b-5p	9.10	hsa-miR-1257	-4.73
hsa-miR-22-3p	9.07	hsa-miR-197-5p	-4.63
hsa-miR-137	8.06	hsa-miR-612	-3.79
hsa-miR-7-1// hsa-miR-7-2// hsa-miR-7-3	6.47	hsa-miR-378f	-3.6
hsa-miR-21-3p	5.43	hsa-miR-34a-5p	-2.39
hsa-miR-4488	4.82	hsa-miR-100-5p	-2.26
hsa-miR-574-5p	4.24	hsa-miR-200a-3p	-2.1
hsa-miR-101-1//hsa-miR-101-2	3.97	hsa-miR-1299	-2.1
… 10 other miRNAs		hsa-miR-10a-5p	-2.1

B) CSC-MDA as compared to pMDA

Upregulated ExomiRs	Fold change	Downregulated ExomiRs	Fold change
hsa-miR-30b-5p	19.05	hsa-miR-28-3p	-13.11
hsa-miR-566	13.78	hsa-miR-128-1-5p	-10.69
hsa-miR-126-3p	9.59	hsa-miR-610	-4.42
hsa-miR-596	8.05	hsa-miR-122-5p	-4.39
hsa-miR-22-3p	7.94	hsa-miR-1257	-4.36
hsa-miR-548a-3p	7.85	hsa-miR-549a	-3.4
hsa-miR-597-5p	7.48	hsa-miR-143-3p	-3.36
hsa-miR-1233-3p	6.15	hsa-miR-30a-3p	-3.32
hsa-miR-30c-2//hsa-miR-30c-1	3.95	hsa-miR-125b-1//hsa-miR-125b-2	-3.26
hsa-miR-7-1//hsa-miR-7-2//hsa-miR-7-3	3.49	hsa-miR-494-3p	-2.91
… 16 other miRNAs		… 415 other miRNAs	

C) Cisplatin-treated CSC-MDA as compared to pMDA

Upregulated ExomiRs	Fold change	Downregulated ExomiRs	Fold change
hsa-miR-126-3p	20.89	hsa-miR-106b-5p	-8.45
hsa-miR-548a-5p	17.52	hsa-miR-1224-5p	-7.35
hsa-miR-548a-3p	17.12	hsa-miR-610	-7.27
hsa-miR-30b-5p	12.52	hsa-miR-30a-3p	-6.42
hsa-miR-939-5p	11.73	hsa-miR-30d-5p	-5.83
hsa-miR-584-5p	10.97	hsa-miR-941	-5.61
hsa-miR-522-3p	10.93	hsa-miR-550a-5p	-5.32
hsa-miR-7-1//hsa-miR-7-2//hsa-miR-7-3	5.43	hsa-miR-320e	-4.7
hsa-miR-139-5p	4.4	hsa-miR-612	-4.24
hsa-miR-181b-1//hsa-miR-181b-2	4.03	hsa-miR-575	-3.72
… 28 other miRNAs		… 399 other miRNAs	

D) Cisplatin-treated CSC-MDA as compared to CSC-MDA

Upregulated ExomiRs	Fold change	Downregulated ExomiRs	Fold change
hsa-miR-208a-3p	7.72	hsa-miR-126-3p	-6.9
hsa-miR-33b-5p	4.52	hsa-miR-378g	-6.13
hsa-miR-584-5p	4.38	hsa-miR-451a	-4.02
hsa-miR-432-5p	3.09	hsa-miR-550a-5p	-3.88
hsa-miR-196a-5p	2.36	hsa-miR-642a-3p	-3.37
hsa-miR-574-5p	2.22	hsa-miR-320e	-3.05
hsa-miR-939-5p	2.22	hsa-miR-769-5p	-3.03
has-miR-221	2.02	mir-30c-2//mir-30c-1	-2.85
		hsa-miR-378f	-2.19
		hsa-miR-206	-2.02

All data were expressed in fold change (FC) for triplicate of independent experiments where $p < 0.05$ regarded as significant different of the respective comparison.

From the list of differentially expressed miRNAs, four exomiRNAs including miR-30a-5p, miR-28-3p, miR-126-3p and miR-941 were found to be significantly dysregulated in CSC-like subpopulations as detected by both panels (**Table 4.3**).

Table 4.3: Regulation of significantly dysregulated exosomal-miRNAs overlapped between both panels:

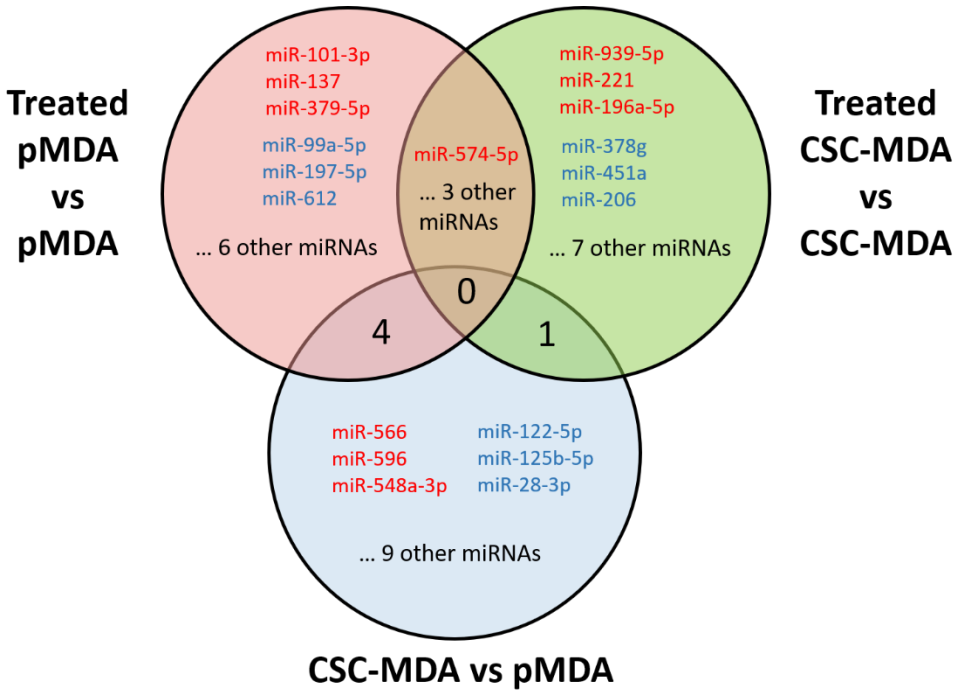
miRNAs	**NGS (FC)**	**p value**	**Nanostring (FC)**	**p value**
CSC-MDA vs pMDA				
hsa-miR-30a-5p	-3.32	0.00088	-2	0.027
hsa-miR-28-3p	-13.11	0.0041	-2.34	0.031
hsa-miR-126-3p	9.59	0.04	3.21	0.012
Treated CSC-MDA vs pMDA				
hsa-miR-941	-5.61	0.044	-2.15	0.016

All data were expressed in fold change (FC) for triplicate of independent experiments where $p < 0.05$ regarded as significant different as compared to pMDA.

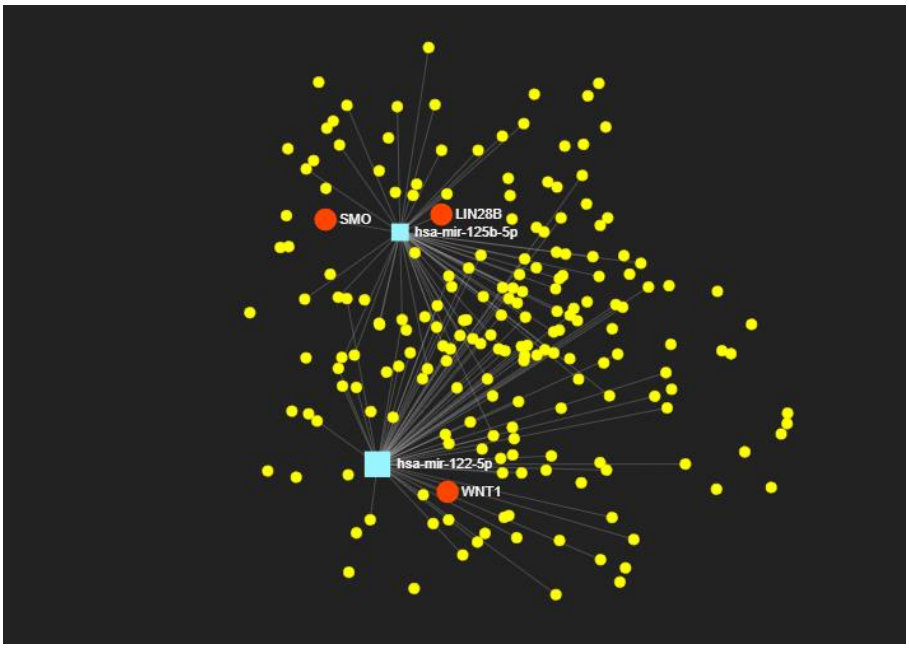
4.5.3 Pathway analysis associated to gene targets of significantly dysregulated exosomal-miRNAs

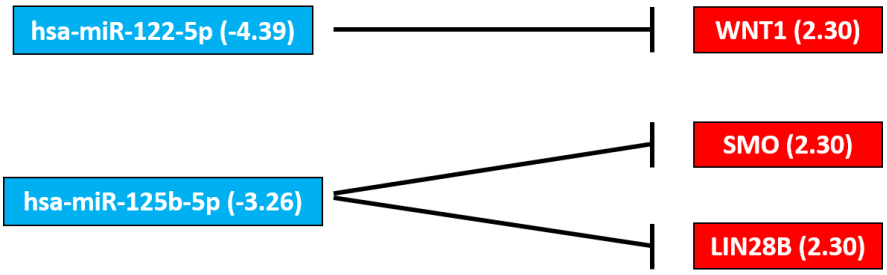
Followed by that, gene ontology (GO) annotation of predicted gene targets of top 10 most dysregulated exomiRNAs as displayed in **Table 4.2** for each comparison were performed via miRNet (http://www.mirne t.ca/) (**Figure 4.12**).

A: Venn diagram of dysregulated exomiRNAs in CSC-MDA and treated subpopulations as compared to their respective controls



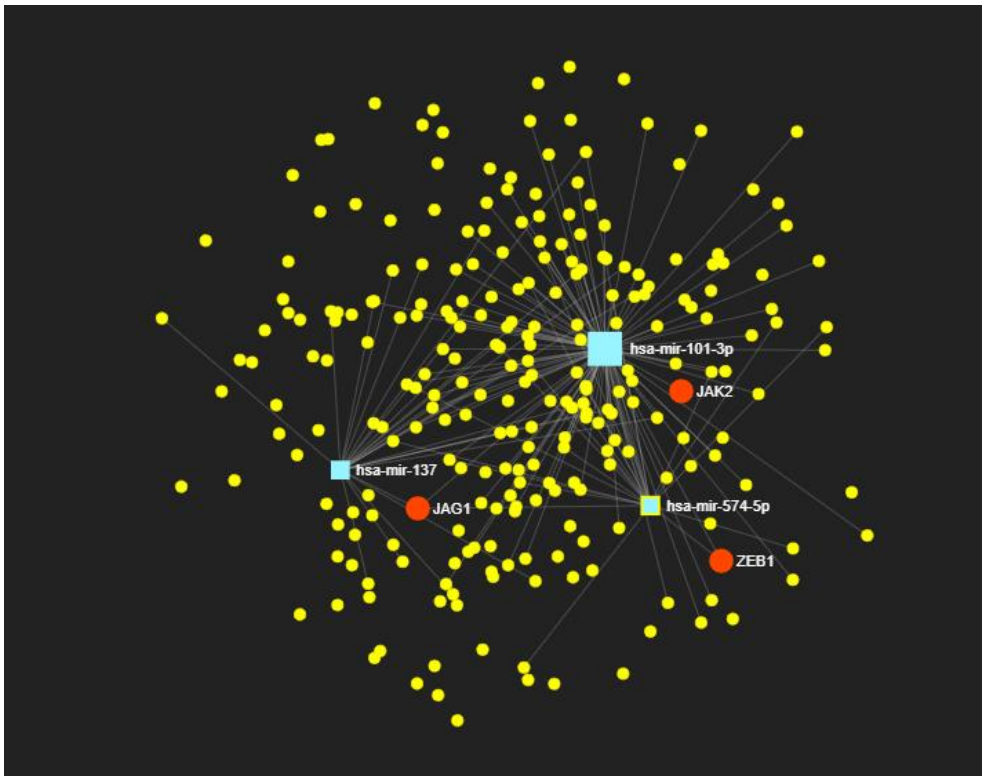
B: CSC-MDA vs pMDA





Annotation	Hits	Corrected p value
Pathways in cancer	195	1.18E-15
Cell cycle	91	1.70E-13
Neurotrophin signalling pathway	86	6.10E-11
Focal adhesion	126	1.65E-10
Chronic myeloid leukemia	56	5.12E-10
Small cell lung cancer	60	5.98E-10
Prostate cancer	64	6.32E-10
HTLV-I infection	121	9.11E-09
Renal cell carcinoma	46	1.95E-08
TGF-beta signalling pathway	59	5.14E-08

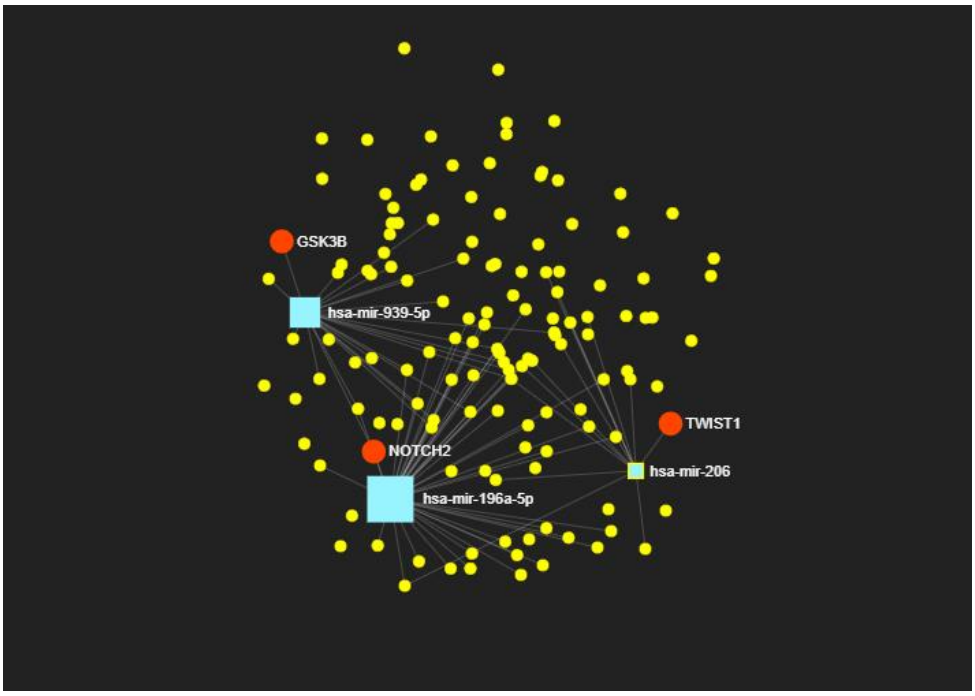
C: Treated CSC-MDA vs pMDA

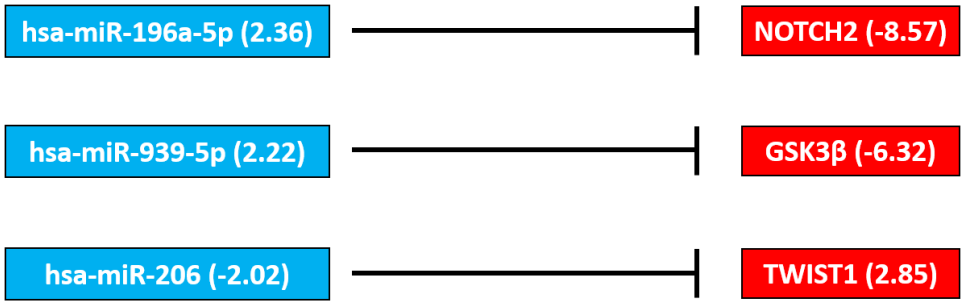




Annotation	Hits	Corrected p value
Pathways in cancer	310	1.76E-16
Focal adhesion	200	1.74E-12
Protein processing in endoplasmic reticulum	129	4.53E-11
Cell cycle	124	7.05E-11
HTLV-I infection	199	6.20E-10
Pancreatic cancer	69	2.69E-08
Colorectal cancer	49	6.46E-08
Neurotrophin signalling pathway	123	6.90E-08
Regulation of actin cytoskeleton	182	9.47E-08
Chronic myeloid leukemia	73	1.48E-07

D: Treated CSC-MDA vs CSC-MDA





Annotation	Hits	Corrected p value
Focal adhesion	98	4.18E-10
Cell cycle	68	5.40E-10
Small cell lung cancer	48	3.80E-09
Prostate cancer	51	3.83E-09
Pathways in cancer	133	2.05E-08
Neurotrophin signalling pathway	64	2.81E-08
Chronic myeloid leukemia	43	5.38E-08
Renal cell carcinoma	37	8.75E-08
Regulation of actin cytoskeleton	83	5.36E-07
Non-small cell lung cancer	32	7.08E-07

Figure 4.12: GO enrichment for predicted gene targets of top 10 most up- and down-regulated exomiRNAs in CSC-MDA and treated subpopulations as compared to their respective controls. (A) Venn diagram analysis showed the number of differentially expressed exomiRNAs that were unique and commonly expressed in CSC-MDA and treated subpopulations as compared to their respective controls. Red and blue text represented up- and down-regulated exomiRNAs respectively. Network of differentially expressed exomiRs-target genes for (B) CSC-MDA vs pMDA, (C) treated pMDA and (D) treated CSC-MDA as compared to their respective controls. Each network consists of three types of

nodes, yellow dot represents annotated target genes of cancer-related pathways, red dot represents selected target genes and light blue square represents individual miRNAs. Expression and correlation of exomiRNAs (in blue box) and their predicted target genes, which were detected to be dysregulated in PCR array (in red box) were presented for each comparison. List of enriched KEGG pathways with their corresponding corrected p-values based on hypergeometric test with the most significant pathways (smallest p value) listed from top down.

Based on the acquired annotation report, 8491, 10557 and 4862 target genes were predicted for the dysregulated exomiRNAs in CSC-MDA vs pMDA and both treated subpopulation relative to their controls respectively. The top 10 enriched KEGG pathways of predicted target genes of dysregulated exomiRNAs in respective comparison revealed their common involvement in 'pathways in cancer', 'cell cycle', 'neurotrophin signalling pathway' and 'focal adhesion'. In addition, dysregulated exomiRNAs in both treated subpopulations as compared to their respective controls were predicted to target on genes correlated to 'regulation of actin cytoskeleton' (**Figure 4.12**).

Notably, in consistent to the prediction, three predicted gene targets (WNT1, SMO and Lin-28 Homolog B (LIN28B)) of downregulated exomiR-122-5p and 125b-5p in CSC-MDA via miRNet (http://www.mirne t.ca/) were detected to be upregulated in CSC-MDA as compared to pMDA, determined via PCR array (**Figure 4.12 & Appendix 3-Table 3**). Moreover, three predicted gene targets (JAG1, JAK2 and Zinc Finger E-Box Binding Homeobox 1 (ZEB1)) of upregulated exomiR-137, exomiR-101-3p and exomiR-574-5p in treated pMDA were detected to be downregulated in treated pMDA as compared to pMDA (**Figure 4.12 & Appendix 3-Table 1**). As for treated CSC-MDA, three of the predicted gene targets of upregulated exomiR-196a-5p, exomiR-939-5p and downregulated exomiR-206 in treated CSC-MDA were detected to be downregulated (NOTCH2 and GSK3β) and upregulated Twist-related protein 1 (TWIST1) in treated CSC-MDA as compared to CSC-MDA (**Figure 4.12 & Appendix 3-Table 2**).

Chapter 5

Discussion

5.1 Characteristics of breast cancer stem cell-like subpopulation in MDA-MB-231

5.1.1 Phenotypic characteristics of MDA-MB-231 breast cancer stem cell-like cells

TNBC is characterised by the absence of hormonal ER and PR with no HER-2 amplification (Liao et al., 2018). It is strongly associated with recurrence of metastatic breast cancer, where metastasis is a major cause of patient morbidity and mortality (André & Zielinski, 2012; Seyfried & Huysentruyt, 2013). Study suggested that occurrence of metastasis in breast cancer is correlated to presence of highly metastatic CSCs (Liu et al., 2010). Apart from that, previous studies demonstrated that breast CSCs are highly resistant to anti-cancer drugs, enabling them to survive through treatment and are capable to reinitiate tumour growth via its self-renewability (Al-Hajj et al., 2003; Ayob & Ramasamy, 2018; Ginestier et al., 2007).

In this study, CSC-correlated characteristics such as metastatic ability, cell cycle pattern and self-renewability of CSC-MDA were evaluated respectively by migration and invasion assay, cell cycle analysis and mammosphere formation efficiency test.

Supporting previous finding, CSC-MDA displayed higher migratory ability as compared to pMDA (**Figure 4.4**). Higher migratory ability of CSC-MDA may be correlated to enriched ALDH1 in CSC-MDA as compared to pMDA (**Figure 4.2 & 4.3**). Previous study showed that ALDH1 is positively regulated migratory ability of TNBC (MDA) and reduced rate of migration in TNBC was observed upon suppression of ALDH1 (Li et al., 2017).

Next, CSC-MDA showed slightly higher but significantly number of cells in G0/G1 phase as compared to pMDA, suggesting its entry of quiescent state, a reversible G0/G1 phase (van Velthoven & Rando, 2019) with slower cell cycle progression in CSC-MDA (**Figure 4.6**).

Cell quiescence enables cells to re-enter cell cycle in response to physiological stimuli due to reversible G0/G1 phase and is often associated with stem cells (van Velthoven & Rando, 2019). For example, in normal mammary cells, isolated slow cycling subpopulation of cells (quiescent state) are found to exhibit stem cell features (Pece et al., 2010). Similar as their non-malignant counterparts, slow dividing CSCs were identified in breast tumours, even found abundantly present in poorly differentiated grade 3 breast cancer (Pece et al., 2010). As previous study demonstrated that slow cycling breast cancer cells were capable to survive through anticancer drug that kill bulk of tumour cells *in vivo* (Naumov et al., 2003), presence of slow cycling CSCs in TNBC can induce resistance to conventional drug treatment. Furthermore, as cisplatin relies on mitotic activity to initiate cytotoxicity through formation of DNA adduct on replicating cells (Eastman, 1983; Tanida et al., 2012), quiescent CSC-MDA may be refractory to the cytotoxic effect of cisplatin, thus less susceptible to the treatment (**Figure 4.5**).

Next, self-renewable breast cancer cells are capable to proliferate and reinitiate tumour growth (Bao et al., 2013). Both CSC-MDA and pMDA are highly self-renewable as evaluated by mammosphere formation efficiency test (**Figure 4.7A**). However, CSC-MDA with higher ratio of CD44/CD24 expression generated morphologically different mammosphere, which were more compact than that of pMDA (**Figure 4.7B**). This may be correlated to higher ratio of CD44/CD24 expression in CSC-MDA relative to pMDA (**Figure 4.2 & Appendix 1**), where previous studies demonstrated positive correlation between CD44/CD24 ratio to tumorigenesis in breast cancer (Al-Hajj et al., 2003; W. Li et al., 2017). Therefore, although both pMDA and CSC-MDA retained mammosphere formation ability, CSC-MDA with higher CD44/CD24 ratio generated more rigid mammosphere as compared to pMDA.

Together, these data showed the slow cycling CSC-like subpopulation is more migratory and capable to generate rigid mammomsphere as compared to its parental cell.

5.1.2 Differentially expressed genes in breast cancer stem cell-like MDA-MB-231

As breast CSC-like cells displayed more migratory phenotype compared to their parental cells, the genotypic characteristics of this subpopulation of cells was investigated via RT^2 Profiler PCR array customised for screening of CSC-correlated genes. Transcriptional analysis demonstrated that 70% of the significantly dysregulated genes ($\geqslant$ 2-fold, $p<0.05$), were upregulated in CSC-MDA as compared to pMDA (**Appendix 3-Table 3**).

Among these genes, KITLG, also known as stem cell factor, was most highly upregulated (by 16-fold) in CSC-MDA (**Appendix 3-Table 3**). As the ligand for c-kit receptor, binding of KITLG to c-kit activates its intrinsic tyrosine kinase activity and promoted tumour growth (Z.-B. Han et al., 2008). Studies showed the contribution of KITLG in tumorigenesis of various cancers such as colon cancer (Yang et al., 2014), uveal melanoma (Lefevre et al., 2004), gliomagenesis (Sun et al., 2006), NSCLC (Levina et al., 2010) and breast cancer (Z.-B. Han et al., 2008), where elevated KITLG was suggested to favour angiogenesis in breast cancer (Z.-B. Han et al., 2008).

On the other hand, KLF17 was the most downregulated gene (by 9.6-fold) in CSC-MDA as compared to pMDA (**Appendix 3-Table 3**). Previous study reported its role as negative regulator for EMT where lower expression of KLF17 was associated with migratory phenotype in breast cancer cell line (Gumireddy et al., 2009). Other than that, discoidin domain receptor 1 (DDR1), a collagen receptor (Shrivastava et al. 1997; Vogel et al. 1997), is the second most downregulated gene (by 3.5-fold) in CSC-MDA as compared to pMDA. Expression of DDR1 is essential for the regulation of migration in breast cancer (Jing et al., 2018). For example, ectopic expression of tumour suppressor DDR1 inhibited migration of DDR1-deficient TNBC (MDA), mediated by phosphoprotein DARPP-32, showing tumour suppressor role of DDR1 in TNBC (Hansen et al., 2006).

In consistent with these previous findings, CSC-MDA in this study with lower expression of KLF17 and DDR1 may contribute to higher migratory ability in CSC-MDA as compared to pMDA (**Figure 4.4**).

Together, these data provide intriguing hints that the pre-treated CSC-MDA have genotypic profile that may enhance its migratory ability as compared to pMDA, supported by the observed higher rate of migration in CSC-MDA.

5.2 Response of breast cancer stem cell-like MDA-MB-231 to cisplatin treatment

5.2.1 Effect of cisplatin treatment on phenotypic characteristic of breast cancer stem cell-like MDA-MB-231

Cisplatin is one of the major drugs used for TNBC treatment (Ozkan et al., 2012). However, as similar to other conventional drugs, cisplatin resistance often developed over time and this remained unsolved (Isakoff, 2010; Oakman et al., 2010). With previously proposed compromised anti-cancer drugs' efficacy on the more aggressive CSCs in TNBC (Kelly M Britton et al., 2011; Y.-H. Wang & Scadden, 2015; M. Zhang et al., 2010), effect of cisplatin on CSC-like subpopulation was evaluated by investigating cisplatin-induced phenotypic characteristics of CSC-like subpopulation in TNBC.

5.2.1.1 Lower apoptotic event in cisplatin-treated breast cancer stem cell-like MDA-MB-231

Firstly, cell cycle pattern and apoptotic event in cisplatin-treated CSC-MDA were evaluated together with cisplatin-treated pMDA for comparison. Cisplatin that formed DNA adduct with replicating DNA induce DNA damage which may activate G2/M phase checkpoint to arrest the cell for the repair of DNA (Demarcq et al., 1994; Sorenson & Eastman, 1988; Zheng et al., 2017). However, prolonged G2/M arrest and unsuccessful DNA repair could later lead to

apoptosis (Basu & Krishnamurthy, 2010; Demarcq et al., 1994; Zheng et al., 2017), represented as sub G0/G1 content (Kajstura et al., 2007; Peitsch et al., 1993).

Rather than being arrested in G2/M phase, treated CSC-MDA showed a portion of cells arrested in S phase after treatment, although, the percentage of arrested treated CSC-MDA in S phase was lower than that of the percentage of arrested treated pMDA in G2/M phase when compared to their respective controls (**Figure 4.6A**). Besides, cisplatin induced significant cell increment in sub G0/G1 after 48 hours of cisplatin treatment on both pMDA and CSC-MDA, but to a greater extent on pMDA (**Figure 4.6B**). Supported by previous finding, $CD24^{-}/CD44^{+}$-expressing CSC-like breast cancer cell displayed lower cell arrest in G2/M phase and lower cell death after radiation treatment than that of non-CSC-like cell (Konge et al., 2018). Furthermore, previous finding demonstrated that upon encountering chemo- or radiation insult, glioma CSCs will initiate DNA repair mechanism, escape from entering G2/M phase (S. Bao et al., 2006). Intriguingly, a study conducted on lung CSCs demonstrated their enhanced ability to implement DNA repairs by arresting cells in the DNA replicating S phase after treated by cisplatin (Yu et al., 2017).

On the other hand, data obtained from Annexin V/PI assay showed that cisplatin was capable to induce different degree of apoptosis on pMDA and CSC-MDA. Parental cells displayed lower number of cells in early apoptosis with more cells trapped in late apoptosis/necrosis as compared to CSC-MDA (**Figure 4.5**). The data suggests that CSC-MDA were more resistant to cisplatin treatment as majority of the CSC-like cells were still undergoing early apoptosis while most of the parental cells had already progressed to late apoptosis/necrosis after exposure to equal dosage of cisplatin for the same duration. Previous study reported that elevated p21 expression can neutralise cytotoxicity of cisplatin via activating Nuclear factor erythroid 2–related factor 2 (NRF2) and accelerating the synthesis of reduced GSH to conjugate with cisplatin (C R R Rocha et al., 2014). In correlation with that, another study demonstrated that higher p21 expression and lower activity of the apoptotic protein, caspase-3 were found in cisplatin-treated CSCs as compared to cisplatin-treated non-CSCs, resulting in higher resistance

of CSCs to cisplatin-induced apoptosis (Sasaki et al., 2018). This observation is in line with apoptotic events induced by cisplatin in pMDA and CSC-MDA, where treated CSC-MDA with lower event detected in sub G0/G1 displayed lower number of cells progressed to late apoptosis/necrosis as compared to treated pMDA (**Figure 4.5**)

Together, these data show that treated CSC-MDA were more tolerant towards cisplatin treatment, indicated by lower events in sub G0/G1 and late apoptosis/necrosis which may be associated with lower proliferation rate of CSC-MDA as compared to pMDA.

5.2.1.2 Potential higher metastatic ability in cisplatin-treated breast cancer stem cell-like MDA-MB-231

Based on the acquired rate of migration and invasion in both treated pMDA and CSC-MDA, cisplatin displayed inhibitory effect on the migratory ability and invasiveness of both treated pMDA and treated CSC-MDA with treated CSC-MDA displayed higher rate of migration and invasion than that of treated pMDA (**Figure 4.4**). These results suggest a hint of higher metastatic property of CSC-MDA as compared to pMDA after cisplatin treatment. In fact, positive correlation of $CD24^{-}/CD44^{+}$ breast CSCs biomarker expression and metastatic ability of breast cancer was previously demonstrated (Uchino et al., 2010; Xu et al., 2015; Yoshihara et al., 2005). However, further validation is needed to confirm this claim due to lack of significance in the anti-metastatic effect of cisplatin on CSC-MDA as compared to pMDA in the current setting.

5.2.1.3 Higher self-renewability in cisplatin-treated breast cancer stem cell-like MDA-MB-231

Cancer relapse is associated with CSCs which equipped with self-renewability and capability to produce daughter cells with differentiation ability to induce regeneration of malignant tumour (Bao et al., 2013). In this study, both the control subpopulations had fully retained their self-renewability. No significant difference of mammosphere formation efficiency was observed between control pMDA and CSC-MDA, but CSC-MDA generated more compact

mammosphere than that of pMDA (**Figure 4.7A**). Interestingly, treated CSC-MDA demonstrated higher self-renewability than that of treated pMDA although both control pMDA and CSC-MDA retained their self-renewability at 100% initially (**Figure 4.7A**). In consistent to previous finding that showed positive correlation between $CD24^-/CD44^+$-expressing breast cancer cells and self-renewability (Al-Hajj et al., 2003), treated CSC-MDA that retained higher stemness than that of treated pMDA displayed higher self-renewability, demonstrated by its capability to generate mammosphere after cisplatin treatment while treated pMDA with relatively lower CSC-like subpopulations and higher non-CSC-like cells failed to generate mammosphere (**Figure 4.7**). This indicates that cisplatin inhibited the self-renewability of CSC-MDA to a lower extent, suggesting CSC-like subpopulation to be more tumorigenic as compared to its parental.

Together, these data show that cisplatin induced lower apoptotic effect, lower suppression on self-renewability and rate of proliferation in $CD24^-/CD44^+$-expressing breast CSC-like cells as compared to their parental cells. This suggests that the presence of breast CSC-like subpopulation plays an important role to induce higher tumorigenesis in breast cancer.

5.2.2 Effect of cisplatin treatment on gene expression in breast cancer stem cell-like MDA-MB-231

In order to uncover the genotypic profile that may contribute to acquirement of high tolerance to cisplatin treatment and high self-renewable properties in treated CSC-MDA relative to treated pMDA, CSC-correlated genes expressed between these treated subpopulations were compared. Remarkably, transcriptional analysis showed that up to 97% of the significantly dysregulated ($\geqslant$2-fold, $p<0.05$) genes were upregulated in treated CSC-MDA as compared to treated pMDA, with only one gene being downregulated in CSC-MDA (**Appendix 3-Table 4**). First of all, gene expression of ALDH1A1 was found upregulated (by 2.79-fold) in treated CSC-MDA as compared to treated pMDA (**Appendix 3 - Table 4**), which was in consistent to higher

protein expression of ALDH1A1 in treated CSC-MDA as compared to treated pMDA (**Figure 4.3A**).

Next, among these genes, a total of four genes are involved in 'Hh signalling pathway' (SMO and PTCH1) and 'JAK/STAT signalling pathway' (JAK2 and receptor-type protein tyrosine phosphatase (PTPRC)) (**Figure 4.10A**).

Upregulation of the members of Hh pathway, SMO and PTCH1 in treated CSC-MDA as compared to treated pMDA may contribute to higher drug resistance and self-renewability in CSC-MDA. In fact, Hh pathway has known to be closely correlated to regulation of CSCs (Sari et al., 2018). For example, many studies showed the role of SMO as a G protein-coupled receptor protein in Hh signalling pathway, responsible for regulation of cell differentiation and self-renewal in normal and cancer stem cells (Koike et al., 2017; Ma et al., 2002; Sari et al., 2018; Sims-Mourtada et al., 2015). In previous breast cancer study, elevated gene and protein expression of SMO in CSCs that was characterised by $CD24^{-}/CD44^{+}$ phenotype, displayed higher tumour initiating ability than that of non-CSCs subpopulation, suggesting the role of SMO in maintaining breast CSCs properties (Wang et al., 2014). Besides, the receptor of Hh pathway, PTCH1, was found overexpressed in recurrent and metastatic breast cancers, where elevated PTCH1 in breast cancer patients was correlated with lymph node metastasis, advanced cancer stages, and more aggressive tumour behaviour (Hasanovic & Mus-Veteau, 2018; Riaz et al., 2018). Not only that, previous study showed contribution of PTCH1 in inducing drug resistance through proton motive force to efflux drugs such as doxorubicin, in contrast to ABC transporters, which use ATP hydrolysis. Indeed, the "reversed pH gradient" formed by the acidic extracellular environment and an alkaline cytosol, a hallmark of malignant cancers (Damaghi et al., 2013), allows PTCH1 to function as an efflux pump specifically in cancer cells, resulting in chemotherapy resistance in cancer (Hasanovic & Mus-Veteau, 2018; Wang et al., 2019). Furthermore, recent study showed the tetraspanin-8 (TSPAN8) induced resistance of breast CSCs to chemotherapeutic agents and enhanced tumour formation in mice, as a result of

interaction of TSPAN8 and PTCH1 that eventually activated SMO and the downstream gene expression (Zhu et al., 2019).

Other than that, JAK2 that correlated to JAK/STAT pathway, was upregulated in treated CSC-MDA as compared to treated pMDA. Activation of JAK/STAT signalling pathway in treated CSC-MDA suggesting its role in maintenance of breast CSCs, enhances metastasis and drug resistance in breast cancer cells. In correlation with CSCs, study reported that the IL-6/JAK2/STAT3 pathway was preferentially active in breast CSCs (characterised by $CD24^{-}$ /$CD44^{+}$ phenotype) as compared to non-CSCs subpopulation whereby the inhibition of JAK2 decreased their number and inhibited their growth in xenografts model (Marotta et al., 2011). Another study showed that JAK2 induced drug resistance and metastasis, indicated by overexpressed JAK2 in drug resistant and metastatic TNBC cells (Balko et al., 2016). Reduced *in vitro* mammosphere formation and *in vivo* tumour growth upon inhibition of JAK2/STAT6 signalling pathway in TNBC cells characterised with high expression of JAK2 suggested the oncogenic effect of JAK2 was through activation of JAK/STAT pathway (Balko et al., 2016).

In addition, ATP Binding Cassette Subfamily B Member 5 (ABCB5) was found to be upregulated (by 2.79-fold) in treated CSC-MDA as compared to treated pMDA, where was also upregulated in CSC-MDA as compared to pMDA (by 2.30-fold) (**Appendix 3-Table 3 & Table 4**), suggesting that ABCB5 was enriched in CSC-MDA subpopulations before and after cisplatin treatment as compared to its parental cells. Given the fact that ABCB5 has been previously found enriched in CSCs in other cancer types and anticancer drug-resistant subpopulations (Cheung et al., 2011; de Waard et al., 2015; Frank et al., 2005; Kugimiya et al., 2015), ABCB5 could be correlated to CSC-like subpopulation in MDA which very likely to contribute to higher resistant to cisplatin, reflected by lower late apoptosis/necrosis detected in treated CSC-MDA as compared to treated pMDA (**Figure 4.5**).

Collectively, these previous findings supported that in this study, the enriched Hh, JAK/STAT signalling pathways and enriched ABCB5 in treated CSC-MDA as compared to treated pMDA

were responsible for higher drug resistance, metastatic ability and better maintenance of stemness in treated CSC-MDA.

Next, to understand how CSC-MDA responded differently from pMDA after cisplatin treatment, CSC-correlated gene expression of treated pMDA and treated CSC-MDA relative to their respective controls were compared (**Figure 4.9**). Most (97%) of the significantly dysregulated genes ($\geqslant$2-fold, $p < 0.05$) that were common between treated subpopulations vs. their respective controls were downregulated, whereas only one gene was upregulated (**Figure 4.9**). This data suggests that cisplatin in general hindered the expression of CSC-correlated genes in both CSC-MDA and pMDA, but to a different extent. For example, approximately 60% of these commonly dysregulated genes were downregulated to a lower extent in CSC-MDA subpopulation than pMDA subpopulation after treatment (**Figure 4.9 & Appendix 3-Table 1 & 2**). The only upregulated gene in both subpopulation after treatment is DLL4 with negligible difference of 0.1-fold between the increment in CSC-MDA (2.85-fold) and pMDA (2.74-fold) as compared to their respective controls. The remaining 36% of commonly dysregulated genes were downregulated to a higher extent in CSC-MDA subpopulation after treatment when compare to pMDA subpopulation (**Figure 4.9 & Appendix 3-Table 1 & 2**). Among these commonly dysregulated genes, a total of six genes were associated with 'Notch' and 'angiogenesis' signalling pathways (NOTCH1, DLL4 and JAG1) as well as 'PDGF signalling pathway' (JAK2, IKBKB and MYC), where these downregulated genes were suppressed to a lower extent in CSC-MDA subpopulation compared to pMDA subpopulation after cisplatin treatment (**Figure 4.10B**).

Lower suppression on the members of Notch signalling pathway such as NOTCH1 and JAG1 in CSC-MDA subpopulation after treatment could be one of the factors that contributes to a better maintenance of CSCs subpopulation and higher drug resistance in treated CSC-MDA than that of treated pMDA. For instance, previous study showed that elevated Notch1, one of the receptors for Notch pathway, was found to enhance ALDH1 levels in breast CSCs via Notch signalling pathway (Zhong et al., 2016). Consistent to previous study, this study shows treated

CSC-MDA with lower reduction of NOTCH1 gene expression displayed higher ALDH1 activity as compared to treated pMDA (**Figure 4.3 & Appendix 3-Table 1 & 2**). Besides, JAG1, a ligand for Notch receptor, was previously found to be upregulated in drug resistant breast cancer cells where CSC-driven drug resistance in ER^+ breast cancer was correlated to the interaction of JAG1 and NOTCH4 in association with Notch signalling pathway (Simoes et al., 2015).

Apart from involving in Notch pathway, these genes are also members of angiogenesis, which is a process for recruitment of new blood vessels (Zetter, 1998). Previous study showed that Jag1 ligand overexpression promoted neovascularization and growth of experimental tumours in mice (Q. Zeng et al., 2005), Not only that, later study demonstrated the role of JAG1 as antagonist for DLL4-mediated Notch signalling within the sprout, resulting in growth of new vessels (Benedito et al., 2009). In this study, cisplatin downregulated gene expression of JAG1 to a lower extent in CSC-MDA subpopulation while elevated DLL4 in both CSC-MDA and pMDA (**Appendix 3-Table 1 & 2**), suggesting cisplatin induced suppression on angiogenesis for both subpopulations but possibly to a lower extent on CSC-MDA.

Besides, lower suppression of MYC, a gene that correlates to PDGF signalling pathway, in CSC-MDA subpopulation after treatment may be one of the causes of higher drug resistance in treated CSC-MDA than that of treated pMDA as compared to their respective controls (**Figure 4.5**). Breast carcinomas are known to express PDGF, a known connective tissue mitogen (Fitzgerald et al., 2001). Previous study showed that the activation of PDGF signalling pathway in MYC-enriched human mammary epithelial (HME) cell where its phenotype and cellular behaviour was known to be very similar to TNBC cell line (MDA) (Prat et al., 2010), can induce a faster and higher Ca^{2+} rises than that of non MYC-enriched HME cells. This rise of Ca^{2+} then triggered higher expression of fatty acid β-oxidation (FAO) (Casciano et al., 2020) and consequently enhanced drug resistance and maintenance of stemness in breast cancer cells (Wang et al., 2018).

Other studies reported that breast cancer patients with elevated PDGF levels had greater degree of metastatic, lower response to chemotherapy and shorter duration of survival (Ariad et al., 1991; Seymour et al., 1993). A recent study also demonstrated correlation of aggressive biological phenotype with PDGF signalling pathway in TNBC by displaying significant upregulation of intracellular PDGFRα in lymph node metastases and asynchronous recurrences (Jansson et al., 2018).

As a side note, Plasminogen Activator, Tissue Type (PLAT) was most downregulated gene discovered in the commonly dysregulated gene for both treated subpopulations (**Appendix 3-Table 1 & 2**). Previous study demonstrated its correlation with anti-apoptosis and tumour proliferation where knockdown of PLAT restored anticancer effect of gefitinib in NSCLC (Yan et al., 2020). In this study, PLAT gene expression is highly suppressed in CSC-MDA and pMDA but to a lower extent in CSC-MDA after cisplatin treatment, suggesting anti-proliferate and induction of apoptosis to both treated subpopulations, but less effective on CSC-MDA, supported by lower event of late apoptosis/necrosis observed in CSC-MDA as compared to pMDA after cisplatin treatment (**Figure 4.5**).

Noteworthy, the data shows approximately 53% of genes were being dysregulated exclusively in CSC-like subpopulation after cisplatin treatment as compared to pMDA subpopulation (**Figure 4.9**). Of these, five genes are correlated to 'Hh signalling pathway' (SMO and Glycogen synthase kinase-3 beta (GSK3β)) and 'angiogenesis' (NOTCH2, DLL1, WNT1 and GSK3β) (**Figure 4.10C**). The Hh pathway correlated-gene expression of SMO was upregulated while GSK3β was downregulated in treated CSC-MDA as compared to its control (**Appendix 3-Table 2**). As mentioned earlier, activation of Hh signalling pathway mediated by SMO is crucial for maintenance of CSCs and reinforces the metastatic ability and self-renewability of breast cancer cells (Wang et al., 2014). On the other hand, previous study demonstrated the activation of GSK3β by adiponectin induced apoptosis and cell cycle arrest in TNBC cell line (MDA), which was accompanied by suppressed intracellular accumulation of β-catenin and its

nuclear activities leading to reduction of cyclin D1 levels, thus demonstrated tumour suppressive role of GSK3β in breast cancer (Wang et al., 2006).

In correlation with Hh signalling pathway, GSK3β is involved in degrading Gli proteins in absence of Hh ligand, subsequently suppresses SHH signalling (Humke et al., 2010), leading to proteolytic cleavage of full-length Gli (GliFL) into C-terminally truncated repressor form, Gli repressor (GliR), which translocates to the nucleus, where it binds to Hh target gene promoters and keeps them switched off (Goetz & Anderson, 2010). Together, upregulation of SMO, a promoter of Hh pathway and downregulation of GSK3β, which is a suppressor of Hh signalling pathway, was favourable for the activation of Hh pathway in treated CSC-MDA.

Besides, genes involved in angiogenesis, including the upregulated DLL1 and WNT1 and downregulated GSK3β and NOTCH2, might enhance aggressiveness of treated CSC-MDA. A previous study showed that DLL1, a Notch ligand, positively regulated migration and invasion abilities with no effects on proliferation and survival of TNBC cell line (MDA), suggesting its role in metastasis (Sales-Dias et al., 2019). Moreover, knockdown of WNT1 which encoded for ligand protein of Wnt signalling pathway displayed lower self-renewability of breast CSCs *in vitro* (Jang et al., 2015b). Besides, inhibition of Wnt1-mediated signalling, expression of cyclin D1 and phosphorylation of β-catenin resulted in enhanced apoptosis, decreased proliferation and spheroid formation (Yin et al., 2011). On the other hand, NOTCH2 encoded for receptor of Notch signalling pathway, when activated, suppressed tumour growth in human breast cancer xenograft. Noteworthy, although activation of Notch2 induced higher vascularisation, the vessels were smaller and comprised a more immature network as compared with Notch4-activated signalling, suggesting its suppressive role in angiogenesis of breast cancer (O'Neill et al., 2007). These data showed that upregulation of DLL1 and WNT1 together with downregulation of GSK3β and NOTCH2 favoured breast cancer progression in treated CSC-MDA.

In agreement with the observed phenotype of cisplatin-treated subpopulations, Notch and PDGF pathways that are positively correlated to breast cancer progression were hindered to a

lower extent in CSC-like subpopulation after treatment. Not only that, Hh and angiogenesis pathways were exclusively enriched in CSC-MDA after treatment, which could be the core pathways contributing to attenuated cisplatin effect including maintaining self-renewability, inhibiting apoptosis and inducing more aggressive phenotype in CSC-like subpopulation after treatment as compared to parental subpopulation. Therefore, genotypic profiles obtained from PCR array supported the observed phenotypes of treated CSC-like subpopulation as more tumorigenic and resistant to cisplatin treatment. This indicates that CSC-like subpopulation played a vital role in acquirement of cisplatin resistance.

5.3 Transcriptomic profiling of intercellular exosomal-microRNAs in triple negative breast cancer cell

Exosome is a nano-sized (10-200nm) endosome-derived vesicle secreted by cells into extracellular space (Val et al., 2017). It was found capable to mediate transfer of miRNAs as a novel mechanism of genetic exchange between cells (Valadi et al., 2007). Besides, sorting of miRNAs into TDEs is an active and highly regulated process, not a passive process that merely projects intracellular miRNAs content (Hessvik et al., 2012; Langevin et al., 2017; Pigati et al., 2010). Thus, enrichment of miRNAs in TDEs does not occur randomly, but is selectively sorted into exosomes. A plethora of evidence indicated that TDE-mediated cell-cell communication can promote tumour initiation, metastasis, and therapy-resistance in cancer cells within the tumour microenvironment (Azmi et al., 2013; Zhang & Grizzle, 2014).

In this study, presence of isolated exosomes from cultured media was first validated via TEM and DLS where exosome expressed both exosomal surface markers CD63 and CD9 with size ranging from 100-200nm (**Figure 4.11**). The obtained results provided confidence that small extracellular vesicles within the exosome size range had been selectively isolated for this study. Followed by that, exomiRNAs from each subpopulation was extracted and profiled using NGS and Nanostring.

As the commonly used technique for miRNA sequencing, NGS has emerged as the gold standard for discovery and quantification of miRNAs and it is an extremely valuable method for *de novo* discovery of novel small RNAs (Baran-Gale et al., 2015). Generation of small RNA sequencing libraries for NGS involved ligation of adapters to both ends of miRNA followed by reverse transcription, template amplification by PCR and size selection of small RNA species. Several of these steps have been shown to introduce biases and artefacts (Coenen-Stass et al., 2018). These biases are sample processing dependent, therefore often unavoidable as they are less readily controlled (C. Wright et al., 2019). On the other hand, Nanostring required no sample amplification with minimum pre-processing step, minimising bias introduced throughout the process. However, Nanostring is limited for detection only for known miRNAs, without the capability to discover unexpected miRNAs. Since each of these panels has its respective advantages and limitations and are involved in different methodologies, aforementioned biases could be mitigated by determining miRNAs that are significantly dysregulated in both panels to allow discovery of potential biomarkers with higher confidence level.

In order to investigate the underlying mechanism enriched by differentially expressed miRNAs from both panels, combined profile of dysregulated miRNAs ($\geqslant$2-fold, $p<0.05$) from both panels was generated (**Table 4.2**). Annotation of signalling pathway based on top 10 most dysregulated exomiRNAs in each MDA subpopulation compared to pMDA was evaluated to investigate their targeted genes and pathways, particularly for tumorigenic pathways (**Figure 4.12**).

5.3.1 Differentially expressed exosomal-miRNAs and the annotated pathways in cancer stem cell-like MDA-MB-231

With CSC-like subpopulation that demonstrated higher tolerance to cisplatin and featured with more aggressive phenotype, profiling of exomiRNAs dysregulation in CSC-like subpopulation as compared to parental cells in response to cisplatin treatment could yield valuable information

that reveal the exomiRNAs-mediated defensive mechanism against anti-cancer treatment associated with CSC-like subpopulation in TNBC.

Prior to cisplatin treatment, the significantly dysregulated exomiR-122-5p and exomiR-125b-5p were predicted to regulate expression WNT1, SMO and LIN28B in CSC-MDA as compared to pMDA (**Figure 4.12B**).

Based on the annotated report in this study, WNT1 was one of the predicted targets of the downregulated exomiR-122-5p in CSC-MDA (**Figure 4.12B**). In agreement with the annotated report, upregulation of WNT1 could be due to lower suppressive effect of the downregulated exomiR-122-5p in CSC-MDA as compared to pMDA (**Figure 4.12B & Appendix 3-Table 3**). Supporting this study, previous finding reported antitumorigenic effect of miR-122 by targeting WNT1 in glioma cancer (G. Wang et al., 2014). In breast cancer, inhibition of WNT1 was found to induce apoptosis, decrease proliferation and spheroid formation in breast CSCs (Yin et al., 2011). Next, exomiR-125b-5p was predicted to target on SMO and LIN28B in CSC-MDA, which was in consistent with the result obtained from PCR array showing upregulated SMO and LIN28B in CSC-MDA as compared to pMDA (**Figure 4.12B & Appendix 3-Table 3**). In fact, SMO was previously found to be a direct target of miR-125b in cancer, regulating tumour proliferation in association with Hh signalling pathway (Ferretti et al., 2008). Furthermore, SMO plays a vital role in regulation of cell differentiation and self-renewal breast CSCs (Wang et al., 2014). On the other hand, expression of LIN28B was hindered by miR-125b-5p in hepatocellular carcinoma, resulted in inhibition of cell growth and cell migration (Liang et al., 2010). Moreover, previous evidence showed that LIN28B promoted the conversion of epithelial cells to a more undifferentiated stage and maintains tumour cells in the stem-like stage (J. Huang et al., 2019; Sato et al., 2017) where LIN28B often found to be overexpressed in poorly differentiated and advanced-stage breast cancer (Xie et al., 2014).

Thus, downregulated exomiR-122-5p and exomiR-125b-5p could be one of the factors that enhanced expression of WNT1, SMO and LIN28B in CSC-MDA as compared to pMDA, which these genes are crucial for maintenance of breast CSCs (Liang et al., 2010; Sato et al., 2017;

Lixia Wang et al., 2014), and may then contribute to higher migratory ability and chemoresistance in CSC-MDA (**Figure 4.4 & 4.5**).

5.3.2 Differentially expressed exosomal-miRNAs and the annotated pathways in parental and cancer stem cell-like MDA-MB-231 induced by cisplatin treatment

In this study, CSC-MDA responded differently from pMDA after cisplatin treatment, where cisplatin-treated CSC-MDA did not only show enhanced resistance to cisplatin but also projected higher tumorigenic potential relative to cisplatin-treated pMDA. In reference to this observation, dysregulation of exomiRNAs profile in CSC-MDA in response to cisplatin treatment may reveal pivotal information that contributes to development of cisplatin resistance in TNBC associated with presence of CSC-like subpopulation. Not only that, the acquired information may be essential for discovery of potential candidate of prognostic biomarkers and therapeutic targets for cisplatin resistance in TNBC.

For instance, the significantly dysregulated exomiR-574-5p, exomiR-137 and exomiR-101-3p in treated pMDA were predicted to regulate expression of ZEB1, JAG1 and JAK2. As for treated CSC-MDA, significantly dysregulated exomiR-196a-5p, exomiR-939-5p and exomiR-206 were predicted to regulate expression of NOTCH2, GSK3β and TWIST1.

Firstly, ZEB1 was the predicted target of the upregulated exomiR-574-5p in treated pMDA (**Figure 4.12C**). In consistent with the annotated report, enhanced exomiR-574-5p may induce suppression on ZEB1 expression, resulted in downregulation of ZEB1 in treated pMDA as compared to pMDA (**Figure 4.12C & Appendix 3-Table 1**). In fact, exomiR-574-5p was found upregulated to a higher extent in treated pMDA than that of treated CSC-MDA which may contribute to higher inhibitory effect on ZEB1 expression in treated pMDA than that of treated CSC-MDA when compared to their respective controls (**Table 4.2A, 4.2D, Figure 4.12C & Appendix 3-Table 1 & 2**). Supporting result obtained in this study, previous finding on TNBC showed that overexpression of miR-574-5p inhibited ZEB1 expression, which may play a part in inhibition of lung metastasis in TNBC (P.-S. Wang et al., 2018). Besides, ZEB1

was found to favour chemoresistance of TNBC by reducing genotoxic drug-induced double-stranded breaks in TNBC (X. Zhang et al., 2018). Treated pMDA that expressed lower ZEB1 than that of pMDA, with higher reduction of ZEB1 relative to treated CSC-MDA when compared to their respective controls suggested possibility of its contribution to lower protection against cisplatin-induced DNA damage that may lead to higher apoptotic event in treated pMDA as compared to treated CSC-MDA (**Figure 4.5**). Next, upregulated exomiR-137 was predicted to target on JAG1, aligning with the downregulation of JAG1 in treated pMDA as compared to pMDA (**Figure 4.12C & Appendix 3-Table 1**). In agreement with this study, previous finding showed JAG1 as one of the target genes of miR-137 and plays a role in regulating cell growth and migration in breast cancer (J.-S. Zeng et al., 2018). Furthermore, JAK2 was the predicted target of upregulated exomiR-101-3p in treated pMDA, aligned with the downregulation of JAK2 in treated pMDA as compared to pMDA (**Appendix 3-Table 1**). Previous study also demonstrated that miR-101-3p was negatively regulating JAK2 in breast cancer, resulted in activation of apoptosis (Lu Wang et al., 2014). With that, downregulation of JAK2 in treated pMDA to a higher extent than that of treated CSC-MDA as compared to their respective controls may be one of the factors triggered higher apoptotic event in treated pMDA (**Figure 4.5**).

Collectively, higher upregulation of exomiR-574-5p in treated pMDA with upregulation of exomiR-137 and exomiR-101-3p solely in treated pMDA relative to treated CSC-MDA as compared to their respective controls may very likely exerted higher suppressive effect on ZEB1, JAG1 and JAK2 in treated pMDA than that of treated CSC-MDA, which may contribute to lower cell proliferation and protection against cisplatin in treated pMDA (**Figures 4.5 & 4.7**).

On the other hand, in treated CSC-MDA, upregulated exomiR-196a-5p and exomiR-939-5p were predicted to target on NOTCH2 and GSK3β respectively (**Figure 4.12D**). In consistent to the prediction, these predicted target genes NOTCH2 and GSK3β were downregulated in treated CSC-MDA as compared to CSC-MDA (**Figure 4.12D & Appendix 3-Table 2**). Previous evidences showed the suppressive role of NOTCH2 in angiogenesis of breast cancer

(O'Neill et al., 2007) and its association with highly differentiated, poorly proliferative breast cancer and positive correlation to higher rate of survival in breast cancer patient (Parr et al., 2004). Besides, GSK3β was found positively correlated to apoptosis and cell cycle arrest in TNBC cell line (MDA) (Wang et al., 2006).

Apart from that, downregulated exomiR-206 was predicted to target on TWIST1 in treated CSC-MDA. Supporting the prediction, TWIST1 was found upregulated in treated CSC-MDA as compared to CSC-MDA (**Figure 4.12D & Appendix 3-Table 2**). TWIST1, a basic helix-loop-helix transcription factor essential for embryonal development as a master regulator of morphogenesis (Z. F. Chen & Behringer, 1995) and EMT in cancer that leads to metastasis (Fu et al., 2011; J. Yang et al., 2004). Furthermore, TWIST1 was found to induce cisplatin resistance in epithelial ovarian cancer (Roberts et al., 2016). More importantly, TWIST1 was correlated to Mucin 1 transmembrane C-terminal (MUC1-C), forming an autoregulatory loop that functions as a master regulator of EMT, maintenance of cell stemness and drug resistance in TNBC (Hata et al., 2019). On the other hand, low expression of miR-206 in lung adenocarcinoma tissues was correlated to increased cisplatin resistance (Q.-Y. Chen et al., 2016).

Taken together, upregulated exomiR-196a-5p, exomiR-939-5p and downregulated exomiR-206 solely in treated CSC-MDA relative to treated pMDA when compared to their respective controls may contribute to downregulation of NOTCH2 and GSK3β with upregulation of TWIST1 solely in treated CSC-MDA. In this study, dysregulation of these genes may attenuate anticancer effect of cisplatin in CSC-MDA, resulted in higher stemness and lower cisplatin-triggered apoptosis in CSC-MDA when compared to pMDA after cisplatin treatment (**Figures 4.2, 4.3 & 4.5**).

5.4 Potential candidates of exosomal-miRNA biomarker for triple negative breast cancer

From this study, a set of exomiRNAs (miR-30a-5p, miR-28-3p, miR-126-3p and miR-941) were detected to be significantly dysregulated in both NGS and Nanostring panels (**Table 4.3**). These identified exomiRs with high confidence were only detected in CSC-like subpopulations with three exomiRNAs being significantly dysregulated in CSC-MDA and one in treated CSC-MDA as compared to their parental cells.

Secretion of exo-miR-30a-5p and miR-28-3p were hindered while exomiR-126-3p was enriched in CSC as compared to pMDA. Previous studies showed that the dysregulation of these miRNAs induced breast cancer progression respectively. For example, miR-30a inhibited breast tumour growth and metastasis through attenuation of lactate dehydrogenase A (LDHA)-mediated Warburg effect (aerobic glycolysis). LDHA is an essential enzyme for regulation of aerobic glycolysis, a hallmark of cancer (Yu et al., 2017). It was often found overexpressed in many human cancers and correlated with poor clinical outcomes (Li et al., 2017). Study conducted by Li et al. (2017) showed that miR-30a-5p was able to inhibit LDHA expression by directly targeting its 3'-untranslated region (3'-UTR). As a result, through inhibition of LDHA, miR-30a-5p diminished glycolysis by decreasing glucose uptake, lactate production, ATP generation, and extracellular acidification rate and increasing oxygen consumption rate in breast cancer cells. They displayed the importance of miR-30a-5p-regulated glycolysis for breast tumour growth and metastasis in both *in vitro* and *in vivo* studies. Furthermore, breast cancer patients too demonstrated negative correlation of LDHA and miR-30a-5p expression. The study suggested role of miR-30a-5p as a tumour suppressor in breast cancer through the inhibition of LDHA. Another finding showed that miR-30a was significantly downregulated in breast cancer cells. It was found to execute its tumour suppressive effect by reducing breast cancer cell viability, migration and invasion, and activating cell apoptosis (Zhang et al., 2017). Previous studies suggested that miR-30a hindered the development of breast cancer by

regulating the expression of the downstream target gene, Notch1 which is an essential gene associated with cancer, particularly breast cancer, cancer cell proliferation and apoptosis, as well as the promotion of migration and invasion (Al-Hussaini et al., 2011; Sharma et al., 2012; Zhang et al., 2017).

CSC-MDA displayed relatively higher cell proliferation with lower late apoptosis/necrosis as compared to pMDA after treatment (**Figures 4.4, 4.5 and 4.7**). In correlation with this, the reduction of NOTCH1 gene expression in CSC-MDA was lower than that of pMDA after treatment, suggesting that downregulation of exomiR-30a-5p in CSC-MDA may have then contributed to lower NOTCH1 suppression in the treated CSC-MDA (**Appendix 3-Table 1 & 2**). Therefore, lower NOTCH1 suppression in treated CSC-MDA may be associated with lower exomiR-30a-5p in CSC-MDA as one of the CSC-correlated mechanisms to attenuate cytotoxic effect of cisplatin.

Next, previous studies showed that the tumour suppressive effect of miR-28 in cancer is associated with nuclear factor erythroid 2-related factor (NRF2). A study showed that NRF2 gene and protein expression were negatively regulated by miR-28 by targeting 3'-UTR of NRF2 to inhibit its gene expression resulting in suppression of mammosphere formation of MCF7 on 3D culture and inhibition on tumour growth (Yang et al., 2011). NRF2 is an important TF in oxidative stress regulation. It binds to antioxidant response elements within promoters of genes encoding detoxification enzymes (e.g., NAD (P) H-quinone oxidoreductase 1 (NQO1)) and induce gene transcription for these enzymes (Yang et al., 2011). Overexpression of NRF2 is associated with human breast carcinogenesis, where increased NRF2 mRNA levels correlated to poor patient outcome for breast cancer (Zhang et al., 2016). In fact, NRF2 promoted TNBC proliferation and migration by increasing RhoA/ROCK pathway signal transduction. Collectively, these studies demonstrated miR-30a and miR-28 may mediate suppression of breast cancer, suggesting CSC-MDA that secreted lower exo-miR-30a and miR-28 in this study may exert lower suppression of breast cancer progression in CSC-MDA.

Secretion of exomiR-126-3p was enriched in CSC-MDA as compared to pMDA. Surprisingly, instead of promoting cancer progression as estimated by its enrichment in CSC-like subpopulation, vast amount of studies displayed tumour suppressive role of miR-126 in cancers such as hepatocellular carcinoma (Bao et al., 2018), lung cancer (Liu et al., 2009), colorectal cancer (Li et al., 2011), gastric cancer (Feng et al., 2010), prostate cancer (Saito et al., 2009) and breast cancer (Alhasan, 2019; Hong et al., 2019).

In other studies, elevated exomiR-126 was correlated to larger tumour size in breast cancer and induced cancer progression in acute myeloid leukemia (AML) as well as NSCLC. A study conducted to investigate crosstalk between adipocyte and breast cancer showed that exomiR-126 increased catabolism of adipocytes, eventually results in transfer of energy to sustain tumour cell invasion (Wu et al., 2019). Besides, Wu et al. (2019) conducted an *in vivo* study which showed the inhibition of miR-126 displayed smaller size of generated tumour in mice. Moreover, rate of proliferation in mice co-injected with miRNA-126-knockdown MDA cells was reduced as compared to those with normal MDA cells. This study suggested that higher exomiR-126 expression in microenvironment plays an important role in breast cancer progression. Intriguingly, a study reported higher expression of exomiR-126 was correlated to bigger TNBC tumour size despite being downregulated intracellularly in TNBC tissues as compared to normal breast tissues (Paszek et al., 2017).

In breast cancer studies, miR-126 was found to negatively modulate breast cancer progression via VEGFA (Alhasan, 2019) and regulator of G-protein signalling 3 (RGS3) (Hong et al., 2019) in ER-positive breast cancer and TNBC respectively. However, a study then displayed exomiR-126 originated from tumour induced blood vessel formation and malignant transformation in chronic myelogenous leukemia cells (Taverna et al., 2014). The contrary role of exomiR-126 as cancer promoter suggested that cellular-miR-126 and exomiR-126 or even just miR-126 itself may have dual functions in cancer. Their study demonstrated tumour promoter role of exomiR-126, justified the enrichment of exomiR-126 in CSC-MDA observed in this study.

In addition, controversial role of miR-126 in cancer was also demonstrated in a study on AML that displayed miR-126 as tumour promoter. Overexpression of miR-126 suppressed apoptosis and increased the viability of AML cells, whereas the opposite effect was observed when endogenous expression of miR-126 was inhibited. Oncogenic role of miR-126 was consolidated through *in vitro* colony-forming/replating assays which displayed enhanced proliferation and colony-forming/replating capacity of mouse normal bone marrow progenitor cells by miR-126. This data showed that miR-126 may play a crucial role in the development of AML (Li & Chen, 2011).

In support to their findings, researchers evaluated expression of miR-126 in serum, exosome and exosome-free serum fractions in NSCLC patients at early and advanced stages, relative to healthy control. They first suggested tumour suppressive role of miR-126 in NSCLC where its downregulation in serum was correlated to advanced stage NSCLC However, miR-126 from exosomes was found to be present in both early and advanced stage NSCLC patients. The incubation of exosomes from early and advanced NSCLC patients induced blood vessel formation and malignant transformation in human bronchial epithelial cells, suggesting potential role of miR-126 as tumour promoter (Grimolizzi et al., 2017).

Next, exomiR-941 was found to be significantly downregulated in treated CSC-MDA as compared to pMDA. Previous studies demonstrated its role as tumour suppressor in breast cancer, gastric cancer and colorectal cancer (Garcia-Vazquez et al., 2017; Kim et al., 2014; Yan et al., 2011). For example, association of cellular miRNAs profile with pathological complete response (pCR) in TNBC patients receiving 5-fluorouracil, adriamycin, cyclophosphamide–cisplatin/paclitaxel neoadjuvant chemotherapy was investigated in previous study. The pCR is defined by the absence of residual disease in breast and lymphatic nodes after cytotoxic treatment, is a parameter used to discriminate between patients with favourable and unfavourable outcomes to neoadjuvant chemotherapy. This study recruited 10 TNBC patients who achieved pCR and 8 without pCR to investigate their cellular miRNAs profile. They then discovered 11 miRNAs being significantly dysregulated in responder relative to non-responder

patients where miR-941 was one of the upregulated miRNAs in responder. This indicated that non-responder with lower expression of miR-941 was less sensitive to neoadjuvant chemotherapy. However, no further functional study was conducted to verify the contribution of miR-941 in TNBC which hindered patients' response to chemotherapy. In consistent with previous miRNA profiling in TNBC patient, secretion of exomiR-941 was significantly downregulated in treated CSC but not in treated pMDA as compared to parental cells (Garcia-Vazquez et al., 2017). This may cause lower susceptibility of treated CSC-MDA to cisplatin as compared to treated pMDA, which is reflected by lower late apoptosis/necrosis in treated CSC-MDA (**Figure 4.4**).

Furthermore, miR-941 was found to exert tumour suppressor function in gastric cancer cells by reducing cell proliferation, invasion and migration of gastric cancer. Study revealed attenuated carcinogenic of miR-914-transfected gastric cancer was correlated to reduced expression of lysine (K)-specific demethylase 6B (KDM6B) mRNA and protein levels. MiR-914-transfected gastric cancer exhibited profound defects in wound healing and matrigel invasion where such a phenotype is supported by the role of KDM6B in promoting EMT (Li et al., 2015). This study suggested tumour suppressor nature of miR-914 in gastric cancer (Kim et al., 2014). In agreement to Kim et al. (2014), study demonstrated hindered cell growth and migration in colorectal cancer cell, associated with miR-941 (Yan et al., 2011).

These previous studies that showed suppression of miR-941 correlated to lower susceptibility to chemotherapy. Thus, cisplatin-treated CSC-MDA that secreted lower level of exomiR-941 is suggested to favour higher cisplatin resistance in TNBC.

Previous study demonstrated that information derived from a cell line study and confirming the observation through investigations in mice and humans seems to be a valid alternative approach for development of circulating biomarkers (Hannafon et al., 2016). In summary, this study provides suggestive evidence for the role of a novel set of determined exomiRNAs, including miR-30a-5p, miR-28-3p, miR-126-3p and miR-941 as potential candidate for development of biomarker to monitor the effectiveness of cisplatin treatment in TNBC. While these detected

exomiRNAs may not themselves be strong predictors of breast cancer alone, these miRNAs could serve as a companion tools following other screening procedures.

Chapter 6

Summary, Conclusion, Limitation and Recommendations for future research

CSCs which are capable of initiating and maintaining tumour growth and have been implicated for resistance and recurrence of neoplasia, such as in the development of cisplatin resistance in human tumour. Cisplatin is one of the major chemotherapeutic drugs used for treating metastatic mammary tumour but often resulted in occurrence of resistant tumour cells. Presence of CSCs may be responsible for the occurrence of cisplatin resistance in TNBC. However, direct evidence of underlying mechanisms induced by triple negative CSCs that might cause development of cisplatin resistance in TNBC is still lacking. Phenotypic and genotypic studies on breast CSCs subpopulation enable their role and correlated genes that contribute to cisplatin resistance to be revealed. Meanwhile, the emerging role of exosomes as intercellular communicating vesicles has aroused considerable attention. Considering the exosomes' ability to modulate drug resistance of breast cancer cells (Mao et al., 2016; Shen et al., 2017; Singh et al., 2014), exosomes may very likely to be a mediator of CSCs to confer defensive potential to breast tumour against cytotoxic insults. Therefore, profiling of altered exomiRNAs expression in breast cancer cells could provide a more direct and informative assessment of the gene regulatory mechanism underlying the resistance to cisplatin.

In light of this, this research aimed to identify the phenotypic characteristics and dysregulation of CSC-correlated genes in triple negative breast CSCs, thereby revealing the key signalling pathways corresponding to cisplatin resistance in association with breast CSCs. Furthermore, exosomes are found to mediate transfer of miRNAs as a novel mechanism of genetic exchange between cells. Therefore, profiling exomiRNAs that are dysregulated in CSCs as compared to parental cells in response to cisplatin treatment could reveal miRNAs that may mediate defensive potential against cisplatin treatment. The key exomiRNAs that are differentially regulated may serve as important biomarkers for prognosis of cisplatin resistance. Likewise,

signalling pathways that are associated with these miRNAs can also be ‘fine-tuned’ to produce more effective treatment strategies.

Slow cycling CSC-MDA pre-treated by cisplatin may be less sensitive to cisplatin treatment that triggers apoptosis via formation of DNA adduct with rapidly replicating cancer cells. Besides, CSC-MDA displayed significantly higher motility than parental cells. In support of the reported migratory phenotype of CSC-MDA, downregulation of the genes KLF17 and DDR1 in CSC-MDA favoured cell migration. Not only that, downregulation of exomiR-122-5p and exomiR-125b-5p in CSC-MDA may enhanced their respective predicted target genes including WNT1, SMO and LIN28B that could facilitate promotion of pre-treatment anti-apoptotic mechanism and migratory potential in CSC-MDA.

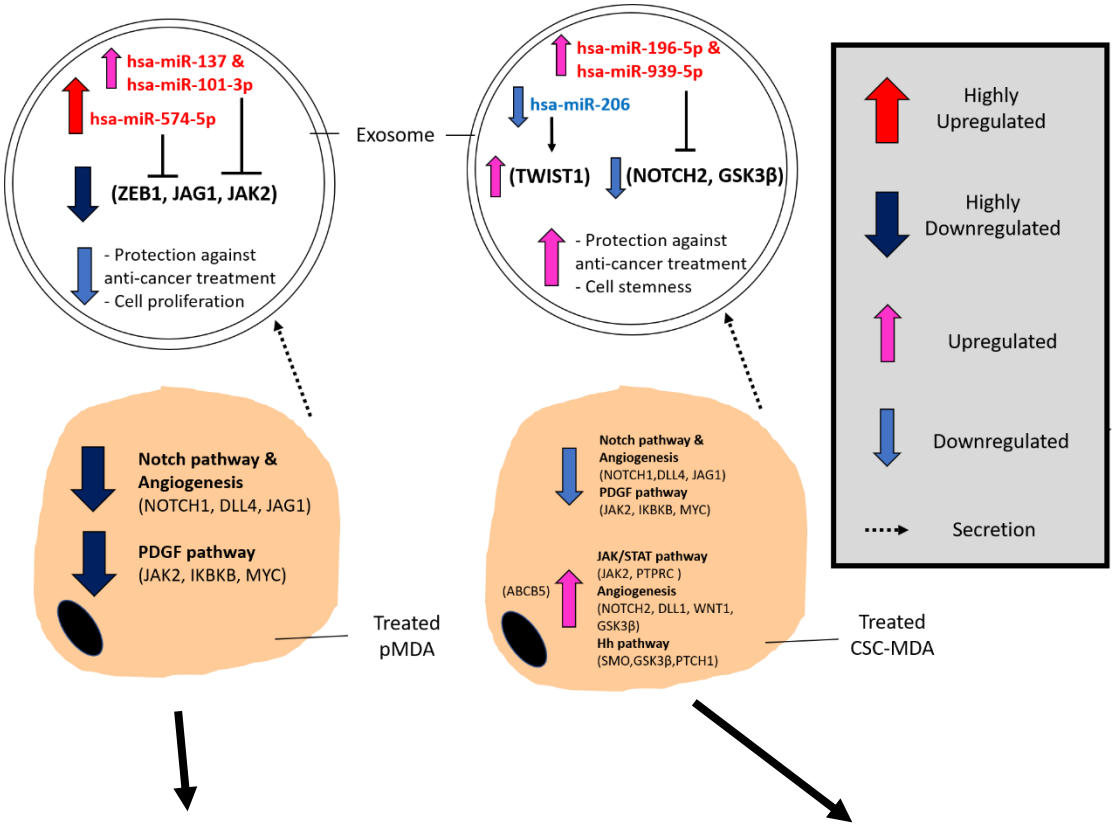
Under the same cisplatin treatment condition (56μM of cisplatin for 48 hours), CSC-like subpopulation displayed relatively lower apoptotic event as demonstrated through lower G2/M phase cell arrest, sub G0/G1 event and lower late apoptosis/necrosis as compared to parental MDA. Furthermore, higher self-renewability and proliferation rate were shown in treated CSC-MDA through the higher number and larger size of generated mammosphere as compared to treated pMDA. Together, these findings showed that CSC-MDA was more tolerant to the cytotoxic effect of cisplatin as compared to pMDA.

Aligning to the reported phenotypic characteristics of CSC-MDA, further genotypic investigation revealed that the underlying mechanisms may contribute to higher cisplatin resistance observed in CSC-MDA, mainly correlated with dysregulation of ABCB5, JAK2, SMO, PTCH1, GSK3β, NOTCH2, DLL1 and WNT1, which most of these genes were associated with JAK/STAT, Hh and angiogenesis signalling pathways. As such, enrichment of these signalling pathways in cisplatin-treated CSC-MDA may be one of the core factors that boosted higher drug resistance and better maintenance of stemness in treated CSC-MDA. This indicates differentially modulated gene expression in breast CSC-like subpopulation plays a vital role in the development of cisplatin resistance in TNBC cell line (MDA).

In support of both the phenotypic and genotypic characteristics of treated subpopulations, upregulation of exomiR-574-5p, exomiR-137 and exomiR-101-3p in treated pMDA may inhibit their respective predicted target genes including ZEB1, JAG1 and JAK2 that could in turn hindered the protection against cisplatin in treated pMDA. In contrast, upregulated exomiR-196a-5p, exomiR-939-5p and downregulated exomiR-206 solely in treated CSC-MDA may contribute to modulation of NOTCH2, GSK3β and TWIST1 that eventually attenuate cytotoxicity of cisplatin, reflected by lower apoptotic event and higher self-renewability in CSC-like subpopulation in MDA.

Additionally, the exomiRNAs which were correlated to cancer progression, including miR-30a-5p, miR-28-3p, miR-126-3p and miR-941 were significantly dysregulated in CSC-like subpopulation as detected by both NGS and Nanostring. These exomiRNAs are suggested to be potential candidate for development of prognostic biomarkers and therapeutic targets of cisplatin-resistant TNBC.

In conclusion, combinations of cisplatin-induced genes and exomiRNAs modulations in CSC-MDA had resulted in lower cytotoxicity effect of cisplatin on CSC-like subpopulation in TNBC as reflected by the assessed phenotypic characteristics (**Figure 6.1**).



Response to cisplatin treatment		
pMDA	Phenotypic characteristic	CSC-MDA
↓ CD24-/CD44+ & ALDH1A1+	**Breast CSCs markers** (Flow cytometry & immunofluorescence microscope)	↓ CD24-/CD44+ & ALDH1A1+
↑ Sub G0/G1 event; Late apoptosis/necrosis	**Anti-apoptosis** (Annexin V/PI & cell cycle analysis)	↑ Sub G0/G1 event; Late apoptosis/necrosis
Cell arrest at G2/M phase (by 13.12%)		Cell arrest at S phase (by 5.98%)
↓ No generated Mammosphere	**Self-renewability** (Mammosphere formation efficiency test)	↓ Number of mammosphere Size of mammosphere

Figure 6.1: Summary of mechanisms, including exomiRNAs and gene dysregulations in pMDA and CSC-MDA in response to cisplatin treatment as compared to their respective controls, followed by comparison of phenotypic characteristics of treated subpopulations.

Nevertheless, there were some limitations of this study which we could not have avoided due to some constraints in commencing the work such as the lack of due a FACS cell sorter in our laboratory. In this study, a simple and time-efficient CSC-like cells isolation method, MACS (Bussolati et al., 2008; Ricci-Vitiani et al., 2007; Sutermaster & Darling, 2019) was chosen to isolate CSC-like cells from pMDA. The CSC-like cells were sorted based on two most prominent biomarkers ($CD24^{-}/CD44^{+}$) only with consideration that prolonged sorting time for MACS-isolation could greatly compromise the viability of sorted cells since this method only allows for selection based on one biomarker at a time. In fact, percentage of CSC-like subpopulations also declined after MACS-isolation when compared to results obtained from surface marker detection via FACS-flow cytometry. We recognise that the use of additional biomarkers such as ALDH1A1 and CD49 into the CSC-like subpopulation isolation may improve accuracy of the sorting and would therefore suggest that FACS sorting method to be used for concurrent sorting of CSC-like subpopulation by using multiple biomarkers to minimise the impact of prolonged cell isolation process on viability of sorted cells.

Furthermore, additional CSC characterisation bioassays such as assessment of OSKM markers (pluripotent markers), a combination of four TFs, namely octamer-binding transcription factor 4 (OCT4), SRY (sex determining region Y)-box 2 (SOX2), KLF4, and MYC or formation of secondary spheroid can be included in future study to ascertain the CSC status of the CSC-like subpopulation prior evaluation of cytotoxic effect of cisplatin treatment.

Two flow cytometry machines were used in this study including BD Accuri C6 flow cytometer in University of Nottingham Malaysia to acquire result for CD24/CD44 surface markers expression and Annexin V/PI apoptosis assay and NovoCyte flow cytometer in University Putra Malaysia to acquire result for ALDH1A1 intracellular marker expression and cell cycle analysis for this study. Change of flow cytometry machine was required as BD Accuri C6 flow cytometer was not available for an extended period of time due to frequent clogging problem and eventually not operable. Due to this limitation, NovoCyte flow cytometer was employed as

an alternative option to acquire ALDH1A1 bioassay and cell cycle analysis while BD Accuri C6 flow cytometer was out of service.

Apart from that, although migration assay was able to determine higher migratory ability in CSC-like subpopulation before treatment, the effect of cisplatin treatment on migratory ability of parental and CSC-like cells was unable to be significantly differentiated. To improve the assessment of cell migratory ability, future research should incorporate cell culture wound closure assay in which a scratch is initiated directly on a confluent cell monolayer. The speed of wound closure and cell migration can be quantified by taking snapshot pictures with a regular inverted microscope at several time intervals (Justus et al., 2014). Such setting enables more detailed cell migratory behaviour to be documented, compensated the weakness of transwell migration assay that showed only the final migrated cell number.

Moreover, invasion assay in this study showed no significant difference of cell invasiveness before and after cisplatin treatment in both parental and CSC-like cells. This could be due to inadequate sensitivity of transwell invasion assay to measure the changes of cell invasiveness after treatment based on merely the final invaded cell number. In future research, invading cell trapping assay can be employed as this assay can segregate and capture living invasive from non-invasive cells (Takino et al., 2018), enable comparative analysis between invasive and non-invasive cells before and after cisplatin treatment.

Furthermore, due to time constraint, current study was restricted to phenotypic and genotypic analysis. Although these data are useful in elucidating the cytotoxic effects of cisplatin in parental and CSC-like MDA, this study lack of protein evidence to confirm the mechanisms. To complement these data, future research can include protein expression study such as western blot analysis to confirm the expression of key regulators.

One subject that remains to be explored is the correlation of the determined exomiRNAs and their specific gene targets in regard to cisplatin resistance in this study. This study had narrowed down the exomiRNAs that could serve as potential prognostic biomarkers for cisplatin

resistance in association to CSC-like subpopulation in TNBC. However, it is undetermined in this study the precise mechanisms induced by these exomiRNAs in regulating cisplatin resistance in TNBC. This can be confirmed in future investigation via miRNAs functional study to help verify the precise mechanisms correlated to the determined exomiRNAs in this study that may involve in modulating cisplatin resistance in TNBC in association with CSC-like subpopulation.

In summary, future research can be conducted to improve efficiency of cisplatin by supressing activity of CSC-like cells through knockdown of essential genes and microRNAs that were determined to be correlated to cisplatin resistance mechanism associated with CSCs. Functional study of dysregulated exomiRNAs secreted by cisplatin-treated CSC-MDA can be carried out to investigate their potential role to enhance or suppress cisplatin resistance in TNBC. A variety of applications can be applied to advance the understanding of these genes and miRNAs, utilising the information for development of biomarker and improve the efficiency of breast cancer treatment.

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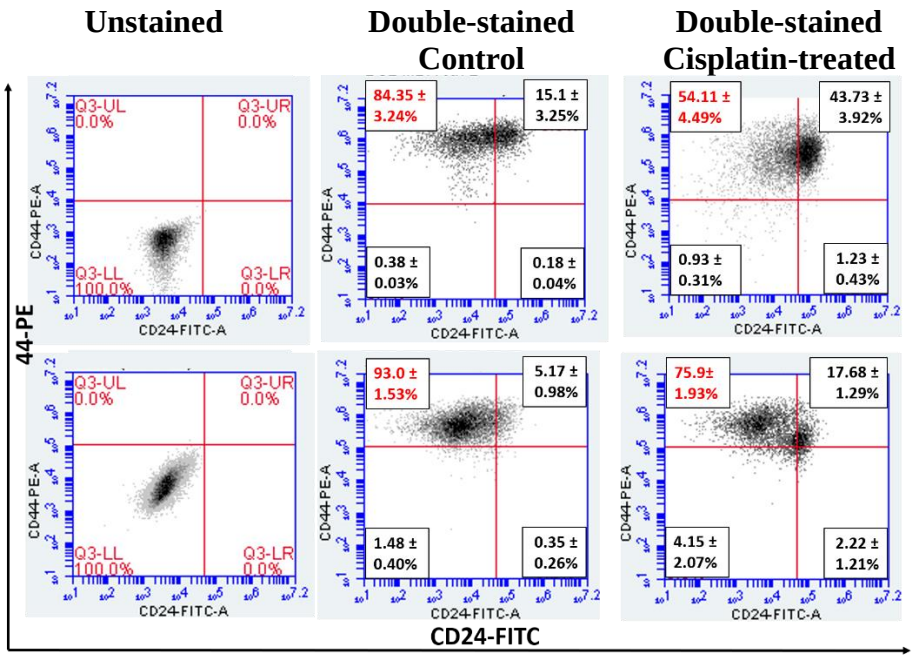
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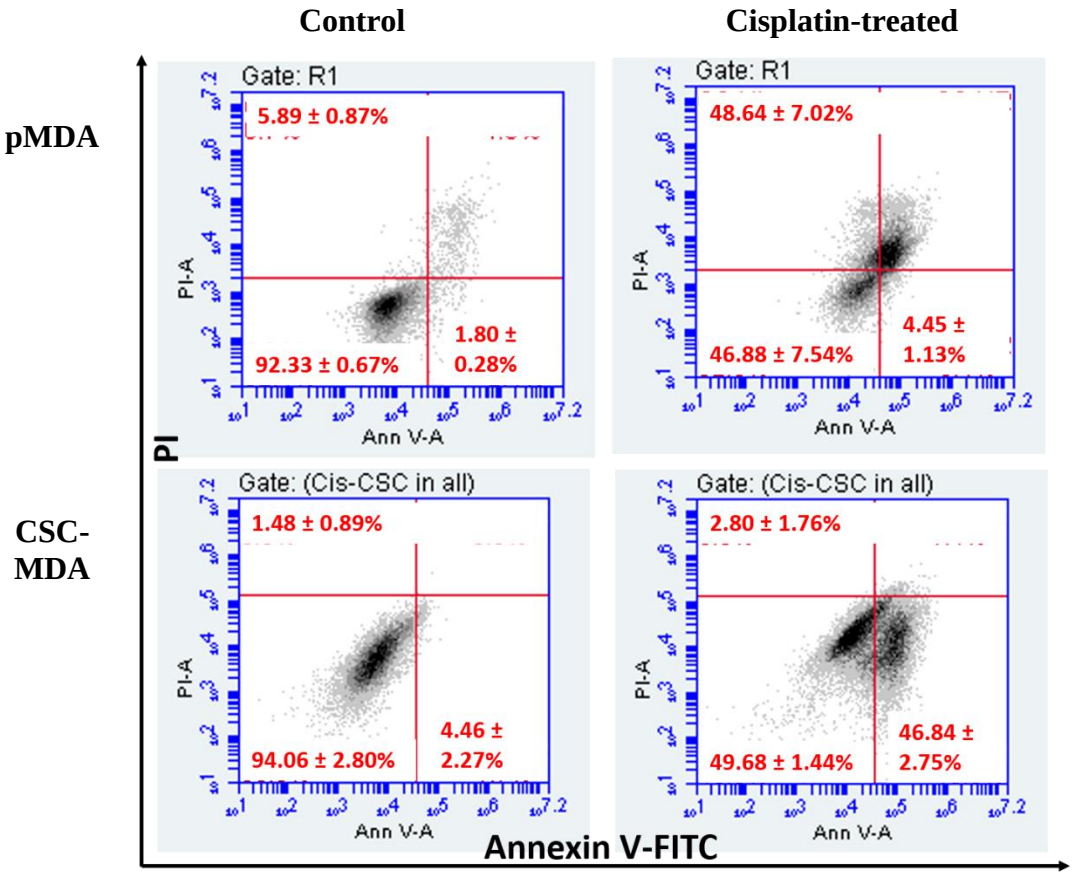
Appendices

Appendix 1



Dot plot of CD24 and CD44 expression in pMDA and CSC-MDA before and after cisplatin treatment determined by FACS flow cytometry. Unstained controls for respective controls was presented, followed by CD24 and CD44-stained controls and cisplatin-treated pMDA and CSC-MDA where percentages of CSC-like subpopulation were presented in red. The values are represented as means ± SEM for triplicate of independent experiments.

Appendix 2



Dot plot of apoptotic event triggered by cisplatin treatment in pMDA and CSC-MDA determined by Annexin V-FITC/PI bioassay. The values are represented as means ± SEM for triplicate of independent experiments.

Appendix 3

Table 1: Significantly dysregulated mRNA at least 2-fold in treated pMDA as compared to pMDA

Refseq	Symbol	Regulation	p-value
Hs.591293	ALCAM	-10.3708	0.00024
Hs.590970	AXL	-3.6584	0.00028
Hs.712050	CD24	-9.2877	0.02189
Hs.374990	CD38	-4.7884	0.01792
Hs.502328	CD44	-3.366	0.00725
Hs.24529	CHEK1	-2.1762	0.00484
Hs.129452	DACH1	-17.2568	0.00031
Hs.631988	DDR1	-15.1365	0.0003
Hs.40499	DKK1	-12.8463	0.00707
Hs.511076	DLL4	2.7376	0.00914
Hs.419815	EGF	-7.6401	0.00113
Hs.542050	EPCAM	-5.0391	0.0031
Hs.446352	ERBB2	-5.693	0.00062
Hs.514038	FLOT2	-4.6928	0.00094
Hs.155651	FOXA2	-4.2726	0.00171
Hs.59368	FOXP1	-34.7805	0.00026
Hs.88556	HDAC1	-2.4179	0.01183
Hs.504609	ID1	-16.7571	2E-06
Hs.597664	IKBKB	-10.9453	2E-06
Hs.482077	ITGA2	-36.079	0.00042
Hs.133397	ITGA6	-36.6162	0.00326
Hs.224012	JAG1	-24.2317	0.01453
Hs.656213	JAK2	-12.7849	3.2E-05
Hs.647667	KLF17	-13.3461	0.00845
Hs.631951	MAML1	-3.6431	0.0017
Hs.306178	MERTK	-28.6492	1.1E-05
Hs.89603	MUC1	-30.6805	0.02495
Hs.202453	MYC	-6.0288	0.01092
Hs.618430	NFKB1	-5.8197	0.00738
Hs.495473	NOTCH1	-13.0746	0.04133
Hs.491582	PLAT	-91.2305	0.02992
Hs.518438	SOX2	4.3413	0.00862
Hs.463059	STAT3	-3.595	0.00095
Hs.409911	TAZ	-6.6513	0.00062
Hs.494622	TGFBR1	-5.3059	0.00377
Hs.503692	YAP1	-8.956	0.01112
Hs.124503	ZEB1	-8.2047	0.00017

Table 2: Significantly dysregulated mRNA at least 2-fold in treated CSC-MDA as compared to CSC-MDA

Refseq	Symbol	Regulation	p-value
Hs.404102	ABCB5	2.85	0.00061
Hs.591293	ALCAM	-9.48	0.00345
Hs.76392	ALDH1A1	2.85	0.00061
Hs.367437	ATM	-4.69	0.00064
Hs.434961	ATXN1	-68.11	0.00134
Hs.590970	AXL	-5.7	0.01193
Hs.473163	BMP7	2.85	0.00061
Hs.712050	CD24	-10.31	0.0308
Hs.374990	CD34	2.31	0.02121
Hs.502328	CD44	-3.04	0.02018
Hs.24529	CHEK1	-2.42	0.01395
Hs.129452	DACH1	-6.98	0.03173
Hs.631988	DDR1	-5.35	0.01089
Hs.40499	DKK1	-2.6	0.0053
Hs.379912	DLL1	2.85	0.00061
Hs.511076	DLL4	2.85	0.00061
Hs.202672	DNMT1	-4.72	0.04223
Hs.419815	EGF	-9.96	0.00896
Hs.76753	ENG	-2.83	0.04357
Hs.542050	EPCAM	-2.35	0.00779
Hs.446352	ERBB2	-4.8	0.00333
Hs.39925	ETFA	-2.35	0.01037
Hs.533683	FGFR2	2.85	0.00061
Hs.514038	FLOT2	-5.73	0.00489
Hs.155651	FOXA2	-7.46	0.01132
Hs.59368	FOXP1	-19.01	0.012
Hs.445733	GSK3B	-6.32	0.01628
Hs.88556	HDAC1	-4.29	0.01772
Hs.504609	ID1	-19.77	0.00031
Hs.597664	IKBKB	-6.47	0.00151
Hs.624	CXCL8	2.39	0.01475
Hs.482077	ITGA2	-30.6	0.00657
Hs.440955	ITGA4	2.85	0.00061
Hs.133397	ITGA6	-21.37	0.00628
Hs.643813	ITGB1	-6.59	0.00336
Hs.224012	JAG1	-17.69	0.00359
Hs.656213	JAK2	-6.87	0.00186
Hs.479754	KIT	2.85	0.00061
Hs.549084	LATS1	-3.59	0.0077
Hs.23616	LIN28B	2.85	0.00061
Hs.631951	MAML1	-3.7	0.00158

Hs.712553	MS4A1	2.85	0.00061
Hs.89603	MUC1	-13.34	0.0064
Hs.202453	MYC	-2.57	0.02943
Hs.25960	MYCN	2.85	0.00061
Hs.635882	NANOG	-3.72	0.02943
Hs.618430	NFKB1	-7.83	0.01179
Hs.709191	NOS2	2.33	0.00529
Hs.495473	NOTCH1	-12.9	0.00707
Hs.487360	NOTCH2	-8.57	0.00153
Hs.514412	PECAM1	-9.37	0.0309
Hs.491582	PLAT	-29.21	0.01069
Hs.249184	POU5F1	-4.33	0.02578
Hs.614734	PROM1	2.85	0.00061
Hs.654514	PTPRC	2.85	0.00061
Hs.642842	SAV1	-2.31	0.04747
Hs.437846	SMO	2.85	0.00061
Hs.48029	SNAI1	13.03	0.00139
Hs.463059	STAT3	-4.17	0.00356
Hs.409911	TAZ	-7.18	0.01304
Hs.494622	TGFBR1	-5.22	0.00595
Hs.644697	THY1	2.85	0.00061
Hs.66744	TWIST1	2.85	0.00061
Hs.745035	TWIST2	2.85	0.00061
Hs.249441	WEE1	-2.94	0.03391
Hs.248164	WNT1	2.85	0.00061
Hs.484047	WWC1	-9.73	0.00662
Hs.503692	YAP1	-6.04	0.02018
Hs.124503	ZEB1	-6.07	0.01736
Hs.34871	ZEB2	-3.24	0.02951

Table 3: Significantly dysregulated mRNA at least 2-fold in CSC-MDA as compared to pMDA

Refseq	Symbol	Regulation	p-value
Hs.480218	ABCB5	2.3043	0.00528
Hs.473163	BMP7	2.3043	0.00528
Hs.502328	CD44	-2.0419	0.02225
Hs.631988	DDR1	-3.5632	0.00152
Hs.533683	FGFR2	2.3043	0.00528
Hs.514038	FLOT2	-2.2825	0.00431
Hs.504609	ID1	2.2962	0.00223
Hs.661108	KITLG	16.0231	0.00651
Hs.647667	KLF17	-4.5738	0.01421
Hs.23616	LIN28B	2.3043	0.00528
Hs.631951	MAML1	-2.0023	0.00131
Hs.712553	MS4A1	2.3043	0.00528
Hs.25960	MYCN	2.3043	0.00528
Hs.437846	SMO	2.3043	0.00528
Hs.66744	TWIST1	2.3043	0.00528
Hs.745035	TWIST2	2.3043	0.00528
Hs.248164	WNT1	2.3043	0.00528

Table 4: Significantly dysregulated mRNA at least 2-fold in treated CSC-MDA as compared to treated pMDA

Refseq	Symbol	Regulation	p-value
Hs.404102	ABCB5	2.79	0.00447
Hs.76392	ALDH1A1	2.79	0.00447
Hs.434961	ATXN1	12.97	0.004403
Hs.473163	BMP7	2.79	0.00447
Hs.479214	CD38	2.79	0.00447
Hs.129452	DACH1	2.08	0.006808
Hs.40499	DKK1	6.71	0.001043
Hs.379912	DLL1	2.79	0.00447
Hs.533683	FGFR2	2.79	0.00447
Hs.514038	FLOT2	-2.05	0.035926
Hs.524134	GATA3	2.79	0.00447
Hs.504609	ID1	2.65	0.017791
Hs.624	CXCL8	8.15	0.00829
Hs.440955	ITGA4	2.79	0.00447
Hs.133397	ITGA6	2.68	0.014163
Hs.656213	JAK2	2.55	0.007727
Hs.479754	KIT	2.79	0.00447
Hs.661108	KITLG	26.08	0.00171
Hs.647667	KLF17	2.79	0.00447
Hs.23616	LIN28B	2.79	0.00447
Hs.712553	MS4A1	2.79	0.00447
Hs.89603	MUC1	4.95	0.002944
Hs.25960	MYCN	2.79	0.00447
Hs.635882	NANOG	2.79	0.00447
Hs.487360	NOTCH2	47.31	0.038399
Hs.514412	PECAM1	2.79	0.00447
Hs.614734	PROM1	2.79	0.00447
Hs.494538	PTCH1	2.79	0.00447
Hs.654514	PTPRC	2.79	0.00447
Hs.437846	SMO	2.79	0.00447
Hs.644697	THY1	2.79	0.00447
Hs.66744	TWIST1	2.79	0.00447
Hs.745035	TWIST2	2.79	0.00447
Hs.248164	WNT1	2.79	0.00447

Table 5: Significantly dysregulated mRNA at least 2-fold in treated CSC-MDA as compared to pMDA

Refseq	Symbol	Regulation	p-value
Hs.404102	ABCB5	6.56	0.000226
Hs.591293	ALCAM	-11.41	0.000188
Hs.76392	ALDH1A1	4.74	0.002723
Hs.590970	AXL	-7.38	0.000123
Hs.473163	BMP7	6.56	0.000226
Hs.712050	CD24	-30.01	0.015241
Hs.374990	CD34	6.33	0.00024
Hs.479214	CD38	-2.34	0.038782
Hs.502328	CD44	-6.21	0.001359
Hs.129452	DACH1	-11.33	0.000337
Hs.631988	DDR1	-19.06	0.000231
Hs.40499	DKK1	-2.61	0.025587
Hs.379912	DLL1	3.94	0.00173
Hs.511076	DLL4	2.97	0.004941
Hs.202672	DNMT1	-3.57	0.007884
Hs.419815	EGF	-10.69	0.000776
Hs.542050	EPCAM	-2.7	0.002837
Hs.446352	ERBB2	-8.9	0.000426
Hs.39925	ETFA	-2.08	0.011455
Hs.533683	FGFR2	6.56	0.000226
Hs.514038	FLOT2	-13.08	0.00019
Hs.155651	FOXA2	-11.1	0.0004
Hs.59368	FOXP1	-23.11	0.000223
Hs.173859	FZD7	-2.75	0.002678
Hs.88556	HDAC1	-4.95	0.000383
Hs.504609	ID1	-8.61	0.000006
Hs.597664	IKBKB	-11.47	0.000002
Hs.624	CXCL8	3.02	0.01497
Hs.482077	ITGA2	-46.15	0.000401
Hs.440955	ITGA4	2.33	0.020292
Hs.133397	ITGA6	-18.62	0.003599
Hs.224012	JAG1	-13.95	0.015669
Hs.656213	JAK2	-6.83	0.000053
Hs.479754	KIT	4.39	0.001006
Hs.661108	KITLG	14.04	0.011484
Hs.647667	KLF17	-6.52	0.010897
Hs.23616	LIN28B	6.56	0.000226
Hs.631951	MAML1	-7.41	0.000027
Hs.306178	MERTK	-23.88	0.000009
Hs.712553	MS4A1	6.56	0.000226
Hs.89603	MUC1	-8.45	0.032484

Hs.202453	MYC	-4.21	0.012408
Hs.25960	MYCN	6.56	0.000226
Hs.618430	NFKB1	-13.27	0.004022
Hs.495473	NOTCH1	-10.82	0.042265
Hs.491582	PLAT	-86.64	0.029768
Hs.466871	PLAUR	-3.68	0.001256
Hs.614734	PROM1	2.38	0.013502
Hs.654514	PTPRC	4.82	0.002155
Hs.437846	SMO	6.56	0.000226
Hs.48029	SNAI1	35.18	0.00174
Hs.518438	SOX2	2.12	0.028248
Hs.463059	STAT3	-5.93	0.000328
Hs.409911	TAZ	-11.96	0.000153
Hs.494622	TGFBR1	-9.12	0.000815
Hs.644697	THY1	4.88	0.00054
Hs.66744	TWIST1	6.56	0.000226
Hs.745035	TWIST2	6.56	0.000226
Hs.248164	WNT1	6.56	0.000226
Hs.503692	YAP1	-4.13	0.008908
Hs.124503	ZEB1	-6.65	0.000135

Appendix 4

NEG_A	ERCC_000	3	6	5	3	2	5	5	7	4	3	5	3
NEG_B	ERCC_000	1	3	1	2	1	1	4	1	1	1	1	3
NEG_C	ERCC_000	3	3	3	2	2	2	2	5	6	3	3	8
NEG_D	ERCC_000	1	1	3	2	3	2	3	2	2	1	2	1
NEG_E	ERCC_000	4	1	5	4	5	3	7	2	1	2	5	2
NEG_F	ERCC_001	1	3	2	2	3	3	3	1	1	5	1	3
NEG_G	ERCC_001	1	1	1	1	1	1	2	1	1	1	1	1
NEG_H	ERCC_001	1	1	1	1	1	1	1	1	1	1	1	1
	Mean	1.875	2.375	2.625	2.125	2.25	2.25	3.375	2.5	2.125	2.125	2.375	2.75
	STDEV	1.246423	1.767767	1.685018	0.991031	1.38873	1.38873	1.92261	2.267787	1.885092	1.457738	1.767767	2.31455
	3 STDEV	3.73927	5.303301	5.055054	2.973094	4.16619	4.16619	5.76783	6.803361	5.655276	4.373214	5.303301	6.943651
Mean+3STDEV		5.61427	7.678301	7.680054	5.098094	6.41619	6.41619	9.14283	9.303361	7.780276	6.498214	7.678301	9.693651

Appendix 5

	pMDA	**Treated pMDA**	**CSC-MDA**	**Treated CSC-MDA**
Percentage of overlapped miRNAs excluding miRNA not existed in Nanostring * (%)	**100**	**100**	**77.05**	**100**
Percentage of overlapped top 25% miRNAs excluding miRNA not existed in Nanostring* (%)	**74.29**	**66.67**	**66.67**	**83.33**
Percentage of overlapped for highly expressed miRNAs (>100 counts) in NGS* (%)	**55.56**	**81.82**	**85.71**	**100**

* Percentage calculated based on overlapped detected miRNAs in NGS and Nanostring (expressed ⩾ 10 counts) as compared to total number of miRNAs detected in NGS.

Appendix 6:

	Mean (10^6 cells)		
	Unsorted cells	$24^-/44^-$ expressing cells	$24^-/44^+$ expressing cells
Viable	16.78 ± 1.85	2.77 ± 0.83	**3.17 ± 0.47**
Dead	1.01 ± 0.37	0.48 ± 0.22	0.47 ± 0.12
Total	17.79 ± 1.90	3.25 ± 0.66	3.64 ± 0.59
% of viable CSC-like cells			**18.73 ± 5.63 %**

Percentage of viable CSC-like cells calculated based on viable $CD24^-/CD44^+$-expressing cells as compared to total unsorted cells. The values are represented as means ± SEM for triplicate of independent experiments.