EPIGENETICS IN CANCER:

BASIC AND TRANSLATIONAL ASPECTS

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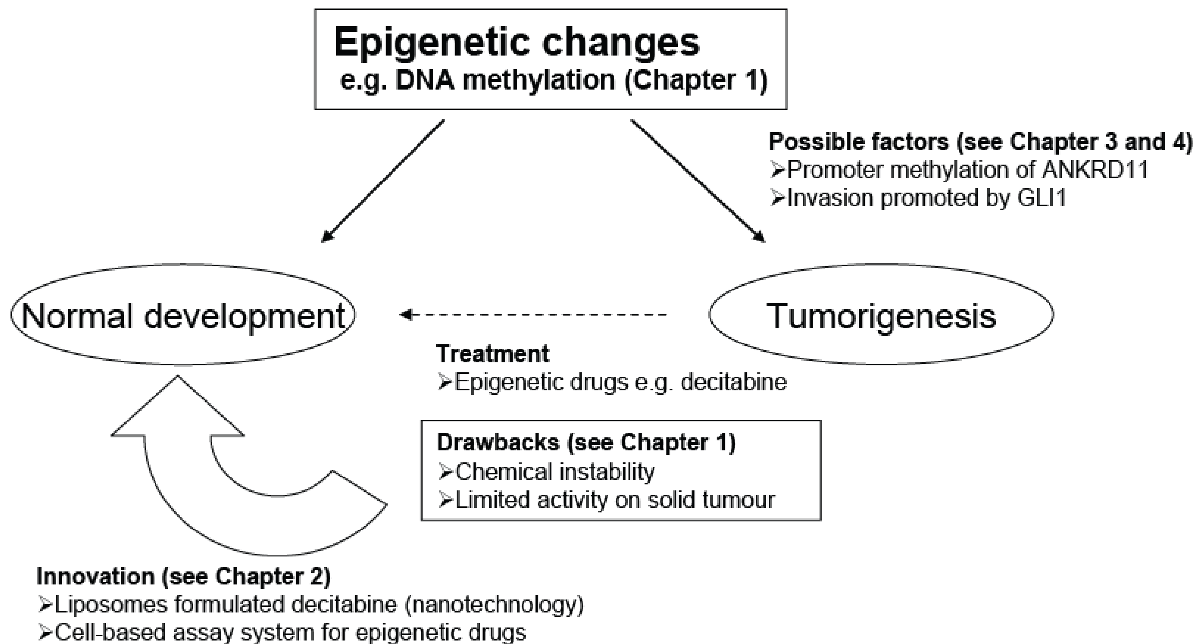
OVERVIEW

Figure: EPIGENETICS IN CANCER: Basic and Translational Aspects



This thesis investigates epigenetics in cancer with particular emphasis on breast cancer. There are two major themes, see Figure above. The first theme relates to the potential for assessing and developing more efficient epigenetic drugs while the second theme investigates mechanism of downregulation of *ANKRD11,* a putative tumour suppressor gene, in human breast cancer. This thesis is in the publication format with Chapters 1 and 3 as published articles, Chapter 2 submitted for publication and Chapter 4 as a manuscript in preparation.

Theme 1: To improve the epigenetic-based therapeutic approach (Chapter 1 and 2)

One of the roles that epigenetics plays in cancer development is the inhibition of transcription of tumour suppressor genes. Chapter 1, published as a review in *Biodrugs*, examines the knowledge of currently available therapeutic approaches related to epigenetic mechanisms such as DNA methylation for cancer treatment. Drug-related issues that could influence the application of therapeutics for clinical use are reviewed and possible developments to improve the clinical use of the drugs explored. Epigenetic-based drugs are emerging as anti-cancer therapies in the clinic. Existing demethylating agents have poor pharmacological properties that limit their clinical use, and the application of nano-based encapsulation to resolve these issues is discussed.

Chapter 2, submitted as an original research article to *Biodrugs*, presents the development and assessment of an assay to allow comparison of epigenetic-related drugs in a high throughput format. Decitabine is encapsulated in a liposomal formulation and the potency of this newly formulated decitabine and existing drugs are effectively compared using the developed assay system. Further development and validation of the assay system and the liposomal formulated decitabine, not included in the submitted manuscript are included as supplementary data.

Theme 2: Investigation of gene silencing mechanism of tumour suppressor ANKRD11 (Chapter 3 and 4)

ANKRD11 is novel gene that was previously characterised in our laboratory, and found to be a putative tumour suppressor gene and a p53-coactivator (Neilsen et al. 2008). Chapter 3, published in *European Journal of Cancer,* investigates the mechanism of downregulation of *ANKRD11* in human breast cancer. This chapter identifies the promoter sequence of *ANKRD11*,

demonstrates the critical region of the *ANKRD11* promoter subjected to DNA methylation, and associates the DNA methylation levels of *ANKRD11* with its gene expression and clinical data. Further analysis of the DNA methylation pattern of this gene revealed a putative GLI1 transcription-factor binding site within the localised region of the promoter that is methylated.

Chapter 4, presented as a manuscript in preparation, further explores the relationship between ANKRD11 and GLI1 in breast cancer. GLI1 is a Hedgehog signalling transcription factor, which has been shown to be involved in breast cancer development. This study analyses the transcriptional activity of *ANKRD11* in the cells overexpressed with GLI1 and quantifies differential expression of these two genes in different stages of breast cancer. Future experiments to confirm and extend these exciting preliminary findings are discussed.

The final chapter of this thesis summarises the findings of these studies and possible future research directions. The impact of these findings for the development of anti-cancer drugs, and the possible role of expression of *ANKRD11* and *GLI1* in breast cancer are highlighted.

DECLARATION

I, Sue Ping Lim, certify that this work contains no material which has been accepted for the award of any other degree or diploma in any university or other tertiary institution and, to the best of my knowledge and belief, contains no material previously published or written by another person, except where due reference has been made in the text.

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CHAPTER 1

Lim et al. (2011). The application of delivery systems for DNA methyltransferase inhibitors. *BioDrugs*; 25 (4): 227-242.

CHAPTER 2

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CHAPTER 3

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"The only reason for time is so that everything doesn't happen at once."

— *Albert Einstein*

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LIST OF ABBREVIATIONS

5′UTR	-	5′ untranslated region
7AAD	-	7-amino-actinomycin-D
A_2O_3	-	Arsenic trioxide
AdoHcy	-	S-adenosylhomocysteine
AdoMet	-	S-adenosylmethionine
ANKRD11	-	Homo sapiens ankyrin repeat domain 11
ATCC	-	American type culture collection
Azacitidine	-	5-azacytidine
C	-	Carbon
-C=O	-	Carbonyl group
CB1954	-	5-(azaridin-1-yl)-2,4-dinitro-benzamide
CES1	-	Carboxylesterase 1
ChIP	-	Chromatin immunoprecipitation
CMV	-	Cytomegalovirus
CpG	-	Cytosine-guanine dinucleotide
DCIS	-	Ductal carcinoma *in situ*
dCK	-	Cytidine/deoxycytidine kinase
Decitabine	-	5-aza-2′-deoxycytidine, Dacogen
DHAC	-	5,6-dihydro-5-azacytidine
DIRAS3	-	GTP-binding protein Di-Ras3
DMEM	-	Dulbecco’s modified Eagle’s medium

DLBCL	-	Diffuse large B-cell lymphoma
DMSO	-	Dimethylsulfoxide
DNMT1	-	Homo sapiens DNA (cytosine-5-)-methyltransferase 1
DNMT3B	-	Homo sapiens DNA (cytosine-5-)-methyltransferase 3B
dNTP	-	Deoxyribonucleotide triphosphate
DOPG	-	1,2-dioleoyl-sn-glycero-3-[phospho-rac-(1-glycerol) sodium salt
DSPC	-	1,2 distearoyl-sn-glycero-3-phosphocholine
DSPE-PEG2000	-	1,2-distearoyl-sn-glycero-3-phosphoethanolamine-N-[amino(polyethylene glycol)-2000] ammonium salt
EGCG	-	(-)epigallocatechin-3-gallate
EMSA	-	Electrophoretic mobility shift assay
EPC	-	Encapsulated papillary carcinoma
EPR	-	Enhanced permeability and retention
ER	-	Estrogen receptor
FCDR	-	5-fluoro-2′-deoxycytidine
FDA	-	Food and Drug Administration
FOXM1	-	Forkhead box protein M1
GLI	-	Glioma-associated oncogene family member
GFP	-	Green fluorescent reporter
H	-	Proton
HA	-	Hyaluronic acid
HDAC	-	Histone deacetylase
HER2	-	Human epidermal growth factor receptor 2

Hh	-	Hedgehog
HPLC	-	High performance liquid chromatography
IBC	-	Invasive breast carcinoma
IC_{50}	-	Half maximal inhibitory concentration
IPC	-	Intracystic papillary carcinomas
LOH	-	Loss of heterozygosity
MALDI-TOF-MS	-	Matrix-assisted laser desorption and ionisation time-of-flight mass spectrometry
mDCIS	-	Micropapillary ductal carcinoma *in situ*
MDS	-	Myelodysplastic syndrome
MIB-1	-	Proliferative index
MLV	-	Multilamellar vesicle
MMP-11	-	Matrix metalloproteinase 11
N	-	Nitrogen
NAD(P)H	-	Nicotinamide adenine (phosphate) oxidase
$-NH_2$	-	Amino group
NKX2.2	-	Homeobox protein Nkx-2.2
NPEOC	-	2'-Deoxy-N4-[2-(4-nitrophenyl) ethoxycarbonyl] group
NTR	-	Nitroreductase
OPN	-	Osteopontin
PAA	-	Poly(acrylic acid)
PAH	-	Poly(allylamine hydrochloride)
PAX6	-	Paired box protein Pax-6

PBS - Phosphate buffered saline

PCR - Polymerase chain reaction

PEG - Poly(ethylene glycol)

PLGA - Poly(lactide-co-glycolide)

PR - Progesterone receptor

PTCH - Transmembrane receptor patched

RT-qPCR - Quantitative real-time polymerase chain reaction

RFP - Red fluorescent reporter

RG108 - 2-(1,3-dioxo-1,3-dihydro-2H-isoindol-2-yl)-3-(1H-indol-3-yl) propanoic acid

RNR - Ribonucleotide reductase

RPS11 - Homo sapiens ribosomal protein S11

RT-PCR - Reverse transcription- polymerase chain reaction

SH - Thiolate

SMO - Smoothened

SuFu - Suppressor of fused

TCEB1 - Transcription elongation factor B polypeptide 1

THU - 3,4,5,6-tetrahydrourine

TMnfsB - Triple-mutated mammalianised nitroreductase B

TXNIP - Thioredoxin interacting protein

VHL - Von Hippel-Lindau disease tumour suppressor

Vorinostat - Suberoylanilide hydroxamic acid, SAHA

WT1 - Wilms tumour gene 1

Zebularine	-	1-(beta-D-ribofuranosyl)-1,2-dihydropyrimidin-2-one
β-actin	-	Homo sapiens actin, beta (ACTB)

CHAPTER 1: INTRODUCTION-THE APPLICATION OF DELIVERY SYSTEMS FOR DNA METHYLTRANSFERASE INHIBITORS

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1.1 Foreword

This introduction was published as a review paper in *Biodrugs*. It introduces the background, mode of action and molecular chemistry of DNA methylation. Epigenetic drugs, specifically demethylating agents, which are responsible for reactivating gene expression that was potentially silenced by DNA hypermethylation and/or chromatin remodelling, are listed. Limitations of existing demethylating agents are summarised and discussed. Modification of existing drugs by drug delivery system is proposed to improve their chemical stability and effectiveness.

1.2 Abstract

DNA methylation, which often occurs at the cytosine residue of cytosine-guanine dinucleotides, is critical for the control of gene expression and mitotic inheritance in eukaryotes. DNA methylation silences gene expression either by directly hindering the access of transcription factors to the target DNA, or through recruitment of histone deacetylases (HDACs) to remodel the chromatin structure to an inactive state. Aberrant hypermethylation of tumour suppressor genes is commonly associated with the development of cancer. A number of anti-cancer agents have been developed that function through demethylation, reversing regional hypermethylation to restore the expression of tumour suppressor genes. Azacitidine and decitabine are used in the clinic, but their applications are limited to myelodysplastic syndrome and other blood-related diseases. Despite the potency of these drugs, their broader clinical application is restricted by cytotoxicity, nonspecific targeting, structural instability, catabolism, and poor bioavailability. Further improvements in the delivery systems for these drugs could overcome the issues associated with inefficient bioavailability, whilst facilitating the administration of combinations of demethylating agents and HDAC inhibitors to enhance efficacy. This review focuses on the current limitations of existing demethylating agents and highlights possible approaches using recent developments in drug delivery systems to improve the clinical potential of these drugs.

1.3 Introduction

DNA methylation was first discovered in calf thymus DNA by Hotchkiss in 1948 (Hotchkiss, 1948). DNA methylation normally occurs on the cytosine residue of the cytosine-guanine dinucleotides (CpG), which are non-randomly distributed across the human genome (Wu and Santi, 1985) and are enriched in short DNA stretches called 'CpG islands' associated with gene promoters (Antequera and Bird, 1993). DNA methylation plays an important role in establishing stable, heritable epigenetic information during normal gene regulation (Jeltsch, 2002). Methylated DNA sequences can promote local chromatin condensation, thereby repressing gene expression through interaction of methylated DNA with methyl-binding proteins, histone methyltransferases, and HDACs (Rodenhiser and Mann, 2006, Holliday and Pugh, 1975, Ellis et al., 2009). Alternatively, DNA methylation can directly inhibit transcription factor recruitment to promoter sequences (Rodenhiser and Mann, 2006, Holliday and Pugh, 1975, Ellis et al., 2009).

Aberrant DNA methylation has been identified as a major factor in carcinogenesis (Bird, 2002, Jones and Baylin, 2002). In particular, hypermethylation of promoters of tumour suppressor genes results in their transcriptional repression, and is a key factor in oncogenic transformation (de Caceres et al., 2004, Esteller et al., 2001, Whitman et al., 2008, Herman et al., 1995). In addition, tumorigenesis can be further driven by DNA hypomethylation at the promoter regions of oncogenes leading to unconstrained expression of their proto-oncogenic products (Grady and Carethers, 2008). In eukaryotes, there are three highly homologous enzymes responsible for DNA methylation, the DNA methyltransferases (DNMTs): DNMT1, DNMT3A, and DNMT3B (Bestor, 2000). Up-regulation of DNMT1 and DNMT3b contributes to

frequent aberrant hypermethylation found in several types of malignancies such as breast and lung cancers (Roll et al., 2008, Biniszkiewicz et al., 2002, Lin et al., 2007).

DNMTs catalyse cytosine methylation by facilitating the transfer of a methyl group from S-adenosylmethionine (AdoMet) to the 5′ carbon of cytidine in CpG dinucleotides (Bestor and Verdine, 1994) (see Fig. 1.1). This transformation involves formation of a covalent bond between the carbon 6 (C6) of the target cytosine with the thiolate (SH) group of DNMT, with subsequent protonation of nitrogen 3 (N3) of cytidine with the glutamyl residue (COOH) of DNMT, and formation of a binding pocket for AdoMet, the methyl donor (Christman, 2002). Addition of the SH-group to the C6 by conjugation leads to an increased electron flow to C5 and protonation at the N3 position of cytosine. The reaction with AdoMet results in the formation of cytosine-enamine, which is then methylated at C5 as shown in Fig. 1.1. During this process, after its methyl group is removed, AdoMet is converted to S-adenosylhomocysteine (AdoHcy). A final beta-elimination, which involves subtraction of a proton (H) from C5, reforms the intact cytidine base to release the DNMT.

Figure 1.1

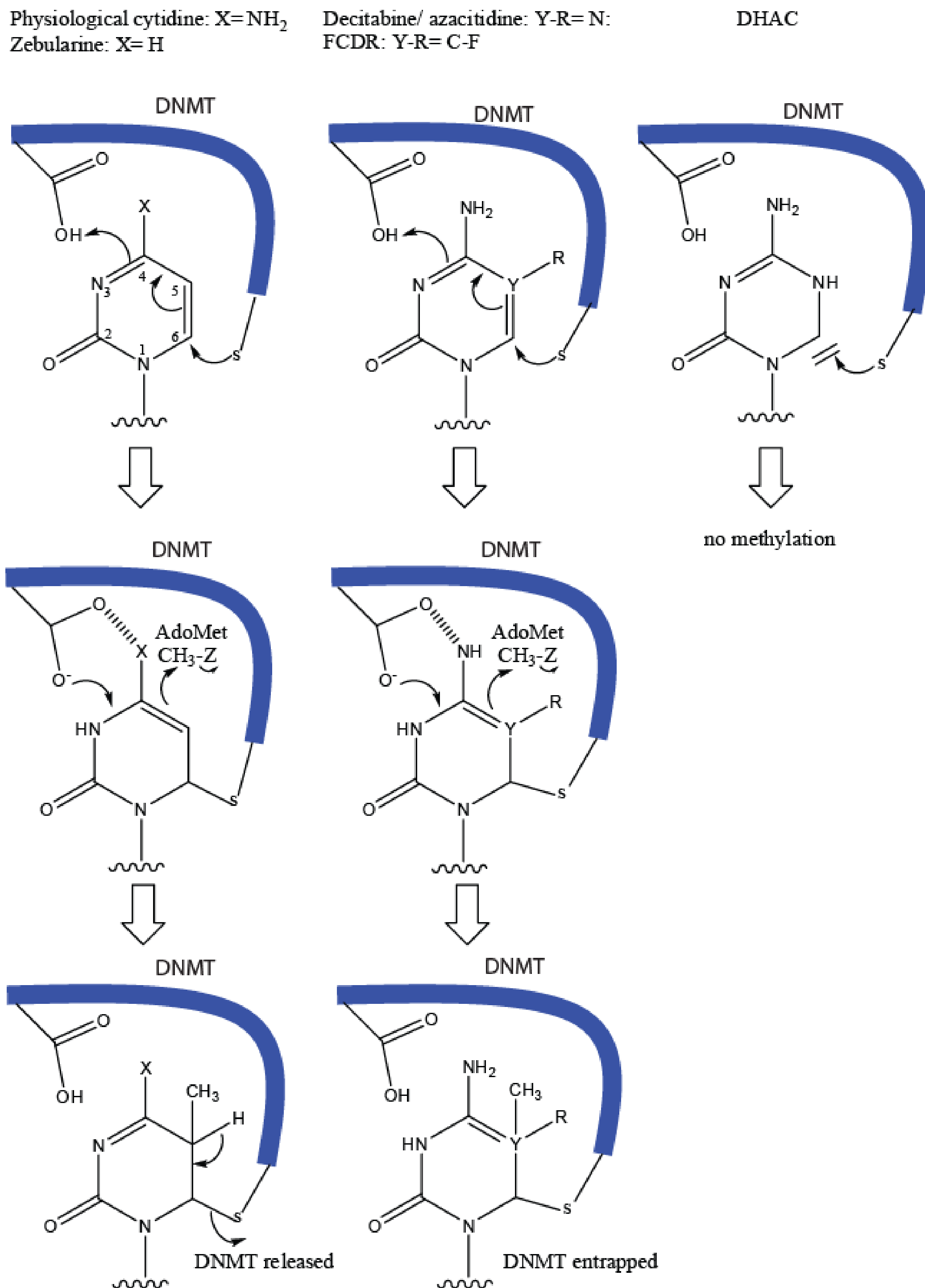

Figure 1.1 The catalytic mechanisms of DNA methyltransferase (DNMT) that occur during the methylation of physiologic cytidine and cytidine analogs (DNMT

inhibitors). (a) First row: DNMT forms a covalent bond between the sulfur atom at the catalytic site of the enzyme and the C6 of physiologic cytidine, 1-(beta-D-ribofuranosyl)-1,2-dihydropyrimidin-2-one (zebularine), decitabine, azacitidine, or 5-fluoro-2′-deoxycytidine (FCDR). The C6 position of 5,6-dihydro-5-azacytidine (DHAC) has an extremely poor affinity toward DNMT, thereby preventing DNMT from completing its catalytic cycle. (b) Second row: The reaction proceeds through an increased electronic flow to C5, with subsequent attack of the methyl group by AdoMet (Z). (c) Third row: Abstraction of a proton from C5 followed by beta-elimination allows reformation of the C5 and C6 double bond and release the DNMT only from the methylated physiologic cytidine or zebularine. Decitabine, azacitidine, and FCDR entrap the DNMT complexes at C6 through the presence of a nitrogen atom (in decitabine and azacitidine) or a fluorine atom (in FCDR) at C5. The covalently trapped DNMTs are degraded and result in reduction of the cellular levels of DNMT, facilitating passive hypomethylation.

Among the mammalian DNMTs, DNMT3A and DNMT3B are responsible for establishing and initiating the *de novo* methylation pattern of certain cell types (Singal and Ginder, 1999). Once the methylation pattern is established, DNMT1 is recruited to hemi-methylated DNA to maintain the pattern of specific methylation (Robert et al., 2002). The DNMT1 can recognize and then bind to hemi-methylated cytidine, resulting in the target cytidine in the complementary strand being exposed to the DNMT1 catalytic pocket (Hermann et al., 2004). The cytosine on the newly synthesized strand is then methylated, resulting in a faithful establishment of the methylation signature. Thus, DNA methylation provides the basis for heritable epigenetic information.

In mammals, the established methylation pattern in somatic cells is typically irreversible and stably transmitted during cell division. In contrast, plants possess glycosylases that provide a mechanism that can actively demethylate DNA (Penterman et al., 2007). Whether similar active demethylation mechanisms exist in mammals is controversial, with available evidence either supporting (Wu and Zhang, 2010) or opposing (Ooi and Bestor, 2008) the involvement of multiple mechanisms for active demethylation. Active, rather than passive, demethylation has been proposed to provide the mechanism for genome-wide demethylation during early embryonic development (Wu and Zhang, 2010). In somatic cells, active DNA demethylation has been suggested to occur at specific gene loci in response to particular stimuli, suggesting dynamic regulation of genes by DNA methylation in addition to provision of long-term epigenetic information (Wu and Zhang, 2010).

As DNA hypermethylation is associated with oncogenesis, the potentially reversible nature of DNA methylation has been exploited as a therapeutic approach for cancer treatment (Ramchandani et al., 1999). This approach uses passive demethylation strategies through targeted inhibition of DNMT1 (Issa, 2007). The inhibition of DNMT can prevent the re-establishment of a preexisting methylation signature on the newly synthesized strand of DNA (Lu et al., 2006). The rationale for using demethylating agents is to reverse epigenetic gene silencing and therefore re-express tumour suppressor genes that will result in restoration of the normal cellular transcriptomic profiles. Restoring the normal controls of cell proliferation can result in increased sensitivity of cancer cells to a range of chemotherapeutic agents such as cisplatin, temozolomide, and epirubicin (Teodoridis et al., 2004).

Current demethylation therapies used in the clinic are based on two major approaches: (i) demethylation analogs derived from cytidine that directly interfere with the normal faithful copying of an established methylation signature following DNA replication; and (ii) agents that interfere indirectly with the process of methylation gene silencing, such as histone inhibitors or cytidine deaminase inhibitors (Amatori et al., 2010). Unfortunately, the clinical benefits of these epigenetic agents are frequently compromised by their poor tumour bioavailability and short half-life. This review will highlight the current development of new systems for drug delivery that have the potential to markedly improve the clinical application of current demethylating agents.

1.4 Demethylating cytidine analogs

Cytidine analogs that mimic the physiologic cytidine nucleotide have been investigated as demethylating agents or DNMT inhibitors since the 1970s (Cihak, 1974, Li et al., 1970). These azanucleosides decrease DNA methylation by inhibiting DNMTs. These therapeutics have anti-tumour potency through the up-regulation of tumour suppressor genes and pro-apoptotic genes that had been previously silenced by hypermethylation during tumorigenesis. Since these drugs nonspecifically demethylate cytidine, global DNA methylation changes using Alu and LINE1 repetitive elements have been used as a biomarker for the activity of these drugs as these elements are distributed throughout the genome (Sellis et al., 2007).

Examples of demethylating cytidine analogs are 5-azacytidine (azacitidine), 5-aza-2′-deoxycytidine (decitabine), 5,6-dihydro-5-azacytidine (DHAC), 5-fluoro-2′-deoxycytidine (FCDR) and 1-(beta-D-ribofuranosyl)-1,2-dihydropyrimidin-2-one (zebularine) (Mai and Altucci, 2009) [Table 1.1]. Cancer cells typically cycle rapidly through cell division, resulting in a more rapid rate of DNA synthesis than normal cells, and this is associated with frequent over-expression of DNMTs (Roll et al., 2008, Rhee et al., 2002, Howell et al., 2010). As a consequence of this high rate of DNA synthesis, cytidine analogs are rapidly metabolized to their active deoxy-triphosphate form and preferentially incorporated into the DNA of cancer cells (Daher et al., 1990).

Table 1.1 Demethylating agents: cytidine analogs

Demethylating agents	Structure[a]	Property	Status	Limitations	References
5-Azacytidine (azacitidine)	Structure 1	Entrap DNMT	US FDA approved for MDS treatment	Unstable in water; toxic; hydrophobic; poor bioavailability	(Piskala and Som, 1964, Cihak, 1974, Biard et al., 1992, Kaminskas et al., 2005a, Caterina et al., 2010, Li et al., 1970, Christman et al., 1983, Borthakur et al., 2008, Creusot et al., 1982, Stresemann and Lyko, 2008, Issa et al., 2004, Lemaire et al., 2008, Patel et al., 2010)
5-Aza-2′-deoxycytidine (decitabine)	Structure 2	Entrap DNMT	US FDA approved for MDS treatment; most potent	Same as 5-azacytidine	(Piskala and Som, 1964, Cihak, 1974, Biard et al., 1992, Kaminskas et al., 2005a, Caterina et al., 2010, Li et al., 1970, Christman et al., 1983, Borthakur et al., 2008, Creusot et al., 1982, Stresemann and Lyko, 2008, Issa et al., 2004, Lemaire et al., 2008, Patel et al.,

					2010)
5-Fluoro-2′-deoxycytidine (FCDR)	Structure 3	Entrap DNMT; reduce affinity of target DNA to DNMT	Also a thymidylate synthase inhibitor; comparable to decitabine	Poor bioavailability; highly toxic (reduce toxicity by combination treatment with THU)	(Boothman et al., 1987, Kaysen et al., 1986, Valinluck and Sowers, 2007, Valinluck et al., 2005, Issa and Kantarjian, 2009, Smith et al., 1992, Klimasauskas et al., 1994, Beumer et al., 2006)
1-(beta-D-ribofuranosyl)-1,2-dihydropyrimidin-2-one (zebularine)	Structure 4	Same as physiologic cytidine to form reversible bond with DNMT; entrap DNMT	Also a bacteriostat and cytidine deaminase inhibitor; hydrophilic; chemically stable; less toxic; orally bioavailability; cancer cell specific; allows long-term treatment	High IC_{50} (50 μmol/L zebularine for equivalent DNMT inhibition of 0.5 μmol/L decitabine)	(Votruba et al., 1973, Zhou et al., 2002, Cheng et al., 2004a, Cheng et al., 2003, Kim et al., 1986, Cheng et al., 2004b, Champion et al., 2010, Kurkjian et al., 2008, Yoo et al., 2008, Ben-Kasus et al., 2005, Mund et al., 2005, Yoo et al., 2004)
5,6-Dihydro-5-azacytidine (DHAC)	Structure 5	Reduce affinity to DNMT	Least potent	Minimal DNA incorporation	(Jones and Taylor, 1980, Sheikhnejad et al., 1999)

a See Fig. 1.2 for chemical structures.

DNMT = DNA methyltransferase; **IC_{50}** = half maximal inhibitory concentration; **MDS** = myelodysplastic syndrome; **THU** = 3,4,5,6-tetrahydrourine; **FDA**: Food and Drug Administration

When incorporated into DNA, the cytidine analogs are targeted by DNMT as hemi-methylated cytidines. A covalent bond is commonly found on the C6 position at the cytosine ring (e.g. decitabine). Due to the differences between the chemical structures of cytidine analogs and physiologic cytidine, the attached DNMTs are entrapped. DNMT-DNA adducts are formed which can trigger a DNA damage response and subsequent G2 phase cell cycle arrest or cytotoxicity (Palii et al., 2008) (Fig. 1.1). Alternatively, the entrapped DNMT is degraded, resulting in an overall reduction in DNMT levels and a passive loss of the inherited methylation pattern (Davidson et al., 1992, Yang et al., 2006).

Among the existing demethylating cytidine analogs, decitabine, azacitidine, FCDR, and zebularine show similar demethylation potencies (see Table 1.1) (Stresemann et al., 2006). However, of these four, the use of FCDR, 5-azacytidine and decitabine as therapeutic agents is limited by their instability in aqueous solution, nonspecific cytotoxicity, susceptibility to inactivation by cytidine deaminase and poor bioavailability (Yoo and Jones, 2006). Among these four analogs, FCDR has the highest nonspecific cytotoxicity, due to the presence of a fluorine atom on the C5 position of the cytosine ring (Fig. 1.2). FCDR is converted to the highly reactive 5-fluoro-2′-deoxyuridine by cytidine deaminase (Beumer et al., 2006). DHAC has been shown to have the least nonspecific cytotoxicity, but this epigenetic drug is also relatively inefficient as a demethylating agent (Sheikhnejad et al., 1999). The poor demethylation activity of DHAC is most likely due to inefficient incorporation into DNA, as the single bond on C5 and C6 of this cytidine analog alters its biophysical properties, leading to the adoption of an unstable structural conformation.

Figure 1.2

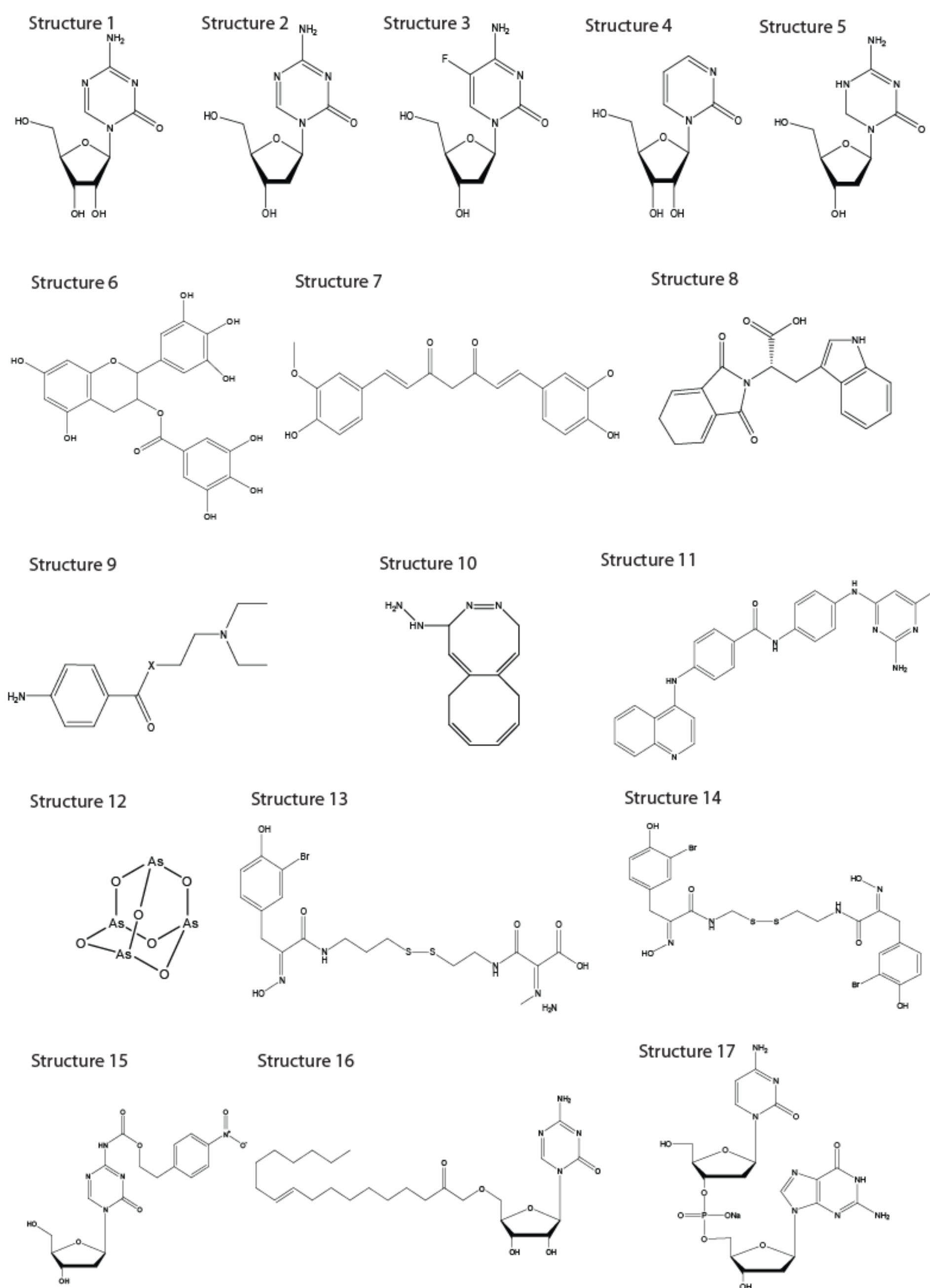

Figure 1.2 Chemical structures of compounds identified as having demethylation potency. The names of cytidine analogs (structures 1–5), non-nucleoside analogs (structures 6–14) or cytidine analogs with modification for enhanced drug delivery (structures 15–17) are listed in Tables 1.1 to 1.3.

Thus far, zebularine, which was identified as a potential demethylating agent in 2004 (Yoo et al., 2004), is the only demethylating cytidine analog with minimal cytotoxicity to normal tissues (Yoo et al., 2008), robust stability in water (Yoo et al., 2004), specificity for cancer cells, (Cheng et al., 2004b) and favourable oral bioavailability (Cheng et al., 2004a). However, zebularine possesses inferior demethylating efficiency compared with decitabine (Mai and Altucci, 2009).

Of the known demethylating agents, decitabine is the most effective demethylating agent. Together with azacitidine, decitabine has been approved by the FDA for treating the blood-related disease myelodysplastic syndrome (MDS) (Kaminskas et al., 2005a, Issa, 2010). In addition to their demethylation effects, azacitidine and decitabine also exhibit a direct cytotoxic effect that causes death of rapidly dividing cancer cells (Issa et al., 2005). Azacitidine and decitabine with favorable safety profiles could improve MDS patient outcomes by eliminating transfusion dependency, improving complete or partial normalisation of blood counts and bone marrow blast percentages, and achieving a reduced risk of transformation to acute leukemia (Kaminskas et al., 2005b, Blum, 2010). However, the cellular uptake of decitabine is poor and this has limited its therapeutic use for any solid tumours (Appleton et al., 2007, Stewart et al., 2009, Cowan et al., 2010). The current available demethylating cytidine analogs and their limitations are summarised in Table 1.1.

1.5 Demethylating non-nucleoside analogs

The substantial nonspecific cytotoxicity of demethylating cytidine analogs is largely due to steric hindrance caused by their incorporation into DNA and the subsequent formation of DNMT-cytidine analog adducts. In fact, this cytotoxic property of cytidine analogs has been exploited in the development of gemcitabine, a cytidine analog with modification on its ribose ring. Gemcitabine is a chemotherapy agent that incorporates into DNA, causing replication errors and as a consequences induces apoptosis in dividing cells (O'Reilly and Abou-Alfa, 2008). In an attempt to overcome these limitations associated with toxicity of demethylating cytidine analogs to normal cells, non-nucleoside compounds that can effectively reactivate silenced genes without incorporation into the DNA have been developed. As they are not incorporated into DNA, these compounds have the added flexibility that they can be modified to enhance hydrophilicity, stability, bioavailability, and aqueous solubility to facilitate oral administration.

These non-nucleoside analogs usually target free DNMTs through a variety of mechanisms, including: (i) non-covalent inhibition of the DNMT1 catalytic site (e.g. RG108) (Mai and Altucci, 2009); (ii) decreasing the affinity of DNMT1 to DNA (e.g. procaine, procainamide, hydralazine, and SG1027) (Castellano et al., 2008, Zambrano et al., 2005, Datta et al., 2009); (iii) suppression of DNMT1 expression (e.g. antisense MG98, curcumin, EGCG, psammaplin A and G); (Pina et al., 2003) or (iv) inhibition of the methyl donor protein, AdoMet (e.g. arsenic trioxide) (Cui et al., 2006) [see Fig. 1.3].

Figure 1.3

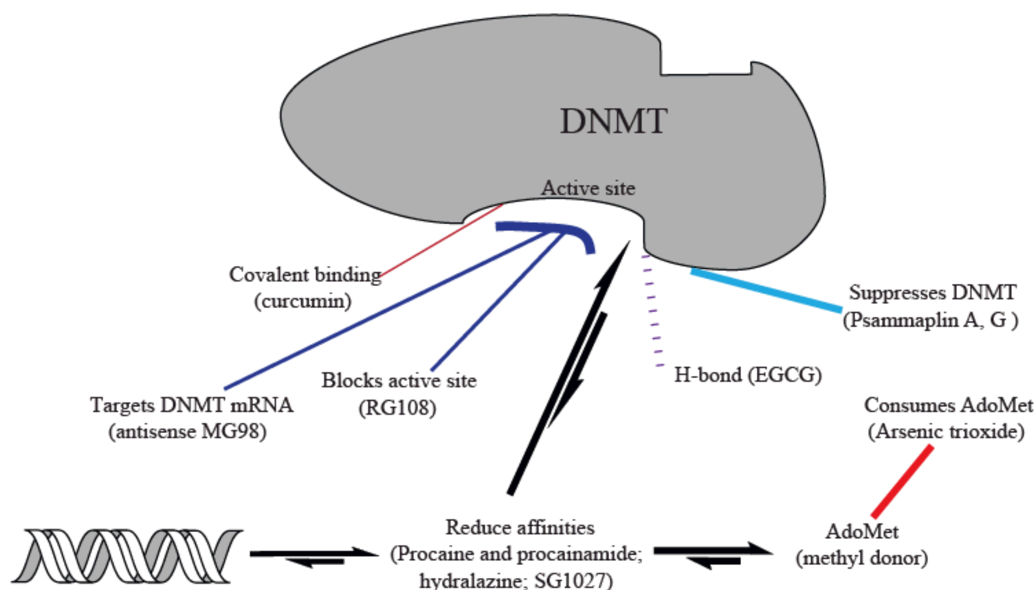

Figure 1.3 Mechanisms of action of non-nucleoside-based demethylating agents. These chemicals can directly inhibit DNMT activity through interaction with the DNMT catalytic site (curcumin and RG108), antisense targeting of the DNMT mRNA (MG98), blocking of the cytosine recruitment site (-)epigallocatechin-3-gallate [EGCG], or suppression of DNMT enzymatic activity (Psammaplin A, G). Alternatively, agents can indirectly inhibit DNMT activity through sequestration of CpG sequences in the target promoter region, thereby decreasing affinity of DNMT for its substrate (procaine, procainamide, hydralazine, or SG1027) or through inhibition of the S-adenosylmethionine (AdoMet) methyl donor (arsenic trioxide).

Among the currently available non-nucleoside analogs, curcumin, RG108, SG1027, As_2O_3 and psammaplin A and G have potencies comparable to decitabine (Suzuki et al., 2010, Datta et al., 2009, Cui et al., 2006, Pina et al., 2003). However, curcumin, RG108, and psammaplin A and G have poor bioavailability while As_2O_3 is highly toxic due to its biophysiologic accumulation. Among these agents, the most promising is the lipophilic quinoline-based demethylating agent SG1027, which has been shown to efficiently demethylate the promoters of the genes *CDKN2A*, *MLHI* and *TIMP3 in vitro* (Datta et al., 2009). The demethylation activity of SG1027 is due to the presence of a quinolinium bisquaternary functional group that allows it to reversibly bind to DNA, therefore blocking DNMT1 activity towards the target DNA (Datta et al., 2009). As a consequence of the polarity of the SG1027 chemical structure, it is stable in aqueous solution and possesses adequate tissue distribution and cellular uptake (Datta et al., 2009). Further preclinical investigations are required to ensure the rapid clinical translation of SG1027. A summary of the mechanism of action of these demethylating non-nucleoside agents are listed in Table 1.2.

Table 1.2 Demethylating agents: non-nucleoside analogs

Demethylating agents	Structure[a]	Property	Status	References
Epigallocatechin-3-gallate (EGCG); phenolic compound of green tea	Structure 6	H-bond with catalytic site of DNMT	Low toxicity; less potent	(Fang et al., 2003, Chuang et al., 2005)
Curcumin; phytochemical of turmeric	Structure 7	Covalently binds to SH-DNMT1	Low toxicity; similar potency as decitabine; phase II trial; poor bioavailability	(Liu et al., 2009, Dhillon et al., 2008)
2-(1,3-dioxo-1,3-dihydro-2H-isoindol-2-yl)-3-(1H-indol-3-yl) propanoic acid, derivative of maleimide (RG108)	Structure 8	First rationally designed DNMT1 inhibitor (from *in silico* screen); blocks active site of DNMT1 without covalent trapping	Minimal toxicity; low solubility	(Mai and Altucci, 2009, Siedlecki et al., 2005, Brueckner et al., 2005, Suzuki et al., 2010)
Procaine (X = O) and procainamide (X = NH); cardiovascular drug	Structure 9	Specific DNMT1 inhibitor; reduce affinity of target DNA to AdoMet and DNMT1	Orally available; less toxic; less potent	(Esteller, 2005, Villar-Garea et al., 2003, Lee et al., 2005, Castellano et al., 2008, Chuang et al., 2005)

Hydralazine; cardiovascular drug	Structure 10	Similar to procaine and procainamide	Minimal toxicity; stable; phase II trial; less potent	(Chatterjee et al., 1976, Cornacchia et al., 1988, Esteller, 2005, Zambrano et al., 2005, Candelaria et al., 2007, Song and Zhang, 2009, Chavez-Blanco et al., 2006, Chuang et al., 2005)
MG98	Antisense oligonucleotides	Specifically targets DNMT1 mRNA	Phase I and II trials; no significant reduction of DNMT activities; cytostatic	(Amato, 2007, Klisovic et al., 2008, Winquist et al., 2006, Stewart et al., 2003)
SG1027; lipophilic, quinoline based	Structure 11	Reversible bind to DNA and reduces affinity of target DNA to AdoMet and DNMT1	Minimal toxicity; stable; good cellular uptake; comparable to decitabine	(Datta et al., 2009)
Arsenic trioxide (As_2O_3)	Structure 12	Consume AdoMet	Comparable to decitabine; poisonous at high concentration	(Shen et al., 1997, Cui et al., 2006, Florea et al., 2007)
Psammaplin G and A; products of marine sponge	Psammaplin G: structure 13	Psammaplin G: identified a specific DNMT inhibitor in cell-	Less toxic; physiologically unstable	(Pham et al., 2000, Pina et al., 2003, Ahn et al., 2008, Simmons et al., 2005)

	Psammaplin A:	free assay system;
	structure 14	Psammaplin A: DNMT and
		HDAC inhibitor

a See Fig. 1.2 for chemical structures.

AdoMet = *S*-adenosylmethionine; **DNMT** = DNA methyltransferase; **SH** = sulphur group.

1.6 Drug delivery systems

From a clinical prospective, the ideal demethylating agent would possess high bioavailability, a restricted mechanism of action, and limited systemic cytotoxicity (Sigalotti et al., 2010). Unfortunately, this is not the case with current demethylating agents under investigation, with the clinical application of these epigenetic drugs restricted by poor uptake into solid tumours, hydrophobicity, rapid clearance, and nonspecific reactivity. It is likely that improvements to the delivery of these agents will facilitate targeted therapy to avoid systemic cell exposure and limit toxicity to non-target cells. Adjustments to the formulation of the delivery system may lead to an optimum delivery of the drug and facilitate the regulation of drug release rates or tissue-specific administration. Recent advances provide avenues to modify existing demethylating agents to enhance their drug delivery and therefore overcome the issues that limit their therapeutic potential. Table 1.3 summarises the current status of modifications to demethylating agents that have been reported to facilitate enhanced drug delivery. However, the potential exists for the application of further approaches to optimize the drug delivery of demethylating agents.

Table 1.3 Modification of demethylating agents to improve their delivery

Origin[a]	Modification[a]	Property	Limitations	References
Decitabine (structure 2)	NPEOC-decitabine (structure 15)	More chemically stable; specific target cells with high level of carboxylesterase	Low solubility; hepatic side effects	(Byun et al., 2008)
Azacitidine (structure 1)	CP-4200 (structure 16)	Improved hydrophilicity; increased cellular uptake	Require activation by plasma membrane lipases	(Brueckner et al., 2010)
Decitabine (structure 2)	S110 (structure 17)	Prevent inactivation by cytidine deaminase	Require activation by cellular phosphodiesterases	(Yoo et al., 2007, Chuang et al., 2010)

a See Fig. 1.2 for chemical structures.

NPEOC = 2'-Deoxy-N4-[2-(4-nitrophenyl) ethoxycarbonyl]-5-aza-2'-deoxycytidine.

1.6.1 Pharmacokinetics of azanucleosides

Of the demethylating agents, decitabine and azacitidine have been the subject of active investigation as lead demethylating agents in clinical trials. Low doses of these azanucleosides are known to be more effective as demethylating agents, as higher doses are cytotoxic (Kantarjian et al., 2007). Current trials using lower dosing schedules, particularly in patients with hematologic malignancies, have shown significant promise for the future use of these azanucleosides as therapeutics (Kantarjian and Issa, 2005). It should be emphasized that there is no formal proof that clinical responses in patients treated with azanucleosides are due to the function of these drugs as demethylating agents rather than their inherent cytotoxicity. However, there are studies showing that a selected hypermethylated gene, *CDKN2B* (p15/INK4B), in MDS is demethylated following continuous low dose treatment with decitabine (Daskalakis et al., 2002, Issa et al., 2004).

It is important to determine the optimal dosage of the azanucleosides that limits any nonspecific cytotoxic effects, but still maintains sufficient delivery to target tissue to achieve a clinical benefit through a demethylation mechanism. A major limitation in achieving this aim with the azanucleoside drugs azacitidine and decitabine is their short half-life and rapid clearance from the systemic circulation. The bioavailability of azacitidine following subcutaneous administration was shown to be superior to intravenous administration (Marcucci et al., 2005). Nevertheless, the half-life of azacitidine is generally short. When measured by liquid chromatography-mass spectroscopy, the mean plasma half-life of azacitidine administrated intravenously (approximately 0.36 hours) is shorter than azacitidine administered subcutaneously (approximately 0.69 hours) (Marcucci et al., 2005). This short half-

life is not solely due to renal elimination of the drug. This study showed the clearance rate of azacitidine (~2445-2791 mL/min) far exceeds the glomerular filtration rate (125 mL/min) and total renal flow (1200 mL/min), suggesting the high clearance also includes non-renal elimination due to instability and metabolism of the drugs (Marcucci et al., 2005). After 6-8 hours the rapid elimination of azacytidine from the plasma resulted in a concentration that was below the threshold of detection by the assay; therefore, the actual amount of the drug corresponding to the *in vivo* pharmacodynamic response could not be determined (Rudek et al., 2005).

A prolonged period of drug delivery rather than bolus administration is required to achieve effective levels of DNA demethylation because of the rapid clearance rates and short half-life of these azanucleosides. As a consequence, various clinical studies have tried to maximize demethylation responses in patients by continuous intravenous or subcutaneous infusion at a low dose of azacitidine or decitabine over several days (O. Odenike, 2007, Samlowski et al., 2005). Analysis of DNA methylation levels of *in vivo* targets showed that such continuous administration of decitabine is successful in producing pronounced demethylation. One study that used both a high bolus dose regimen and a prolonged period of decitabine administration (20 mg/m^2 intravenously over 1 hour daily for 5 days) demonstrated significant anti-MDS potency with an acceptable toxicity profile (Kantarjian et al., 2007). This suggests prolonged low dose treatment, in contrast to the high dose bolus administration, is not associated with significant non-target toxicity.

So far, subcutaneous administration has provided better bioavailability, and the prolonged period of drug delivery has overcome the issue of reduction of active

drugs. However, optimization of the drug is required to allow less invasive (i.e. oral) administration, which could improve the clinical response and reduce the unwanted adverse effects caused by the degraded drugs. The limited oral bioavailability, short plasma half-life and rapid clearance of these demethylating agent-cytidine analogs is due to two major factors: (i) the instability of the structures in an aqueous solution, and in particular in a physiologic environment such as gastric acid; and (ii) the high affinity of these drugs to cytidine deaminase, resulting in subsequent metabolism to uridine analogs.

1.6.2 Inactivation of the drugs

Several different processes (e.g. metabolism, hydrolysis, and degradation) contribute toward the inactivation of the azanucleosides (Marcucci et al., 2005). The azanucleoside compounds are prone to catabolism by the enzyme cytidine deaminase in the liver and spleen (Chabot et al., 1983). As a consequence, the demethylating cytidine analogs are converted to inactive uracil analogs by deaminating the amino group (C-NH_2) on the C4 position of the cytosine ring to a carbonyl group (C=O) (Chabot et al., 1983). For example, the *in vivo* clearance of decitabine and azacitidine was shown to exceed the glomerular filtration rate, which suggests rapid inactivation by cytidine deaminase to a uridine form and therefore the ability to inhibit DNA methylation is lost before renal elimination (Stresemann and Lyko, 2008). The demethylating agent zebularine, which also has the properties of a cytidine deaminase inhibitor due to the absence of an amino group on the C4 position, has been used together with other demethylating agents such as decitabine to maintain the plasma levels of the demethylating agents *in vivo* (Lemaire et al., 2009).

In an attempt to improve the stability and reduce the cytotoxicity of decitabine, Yoo et al (Yoo et al., 2007) generated a derivative of decitabine termed S110 by modification of the structure with the addition of short oligonucleotides. S110 is rapidly converted to decitabine after degradation of the oligonucleotide linkages by cellular phosphodiesterases (Yoo et al., 2007, Lavelle et al., 2010), and has shown *in vivo* demethylation potency similar to decitabine (Chuang et al., 2010). Although S110 did not improve the stability or reduce the cytotoxicity of decitabine, the use of S110 oligonucleotides was shown to protect decitabine from inactivation by cytidine deaminase *in vivo* (Lavelle et al., 2010).

Although treatments using demethylating cytidine analogs in combination with oligonucleotides and cytidine deaminase inhibitors (e.g. zebularine) are feasible approaches, there is still a lack of a strategy to overcome the therapeutic limitation of cytidine analogs caused by *in vivo* metabolism. The protection of the active form of demethylating agents using biodegradable polymers is discussed below and represents an alternative approach to increase the half-life of the drugs. The application of a drug delivery system can possibly prevent the inactivation of the drug by cytidine deaminase by encapsulating the drug in a suitable material. This usually involves fabrication of a drug vehicle with natural or synthetic polymers (Pannier and Shea, 2004).

1.6.3 Instability of the drugs

The instability of demethylating agents in aqueous solution under physiologic conditions, which alters the effective concentration of drugs, is a significant challenge to their clinical administration as cancer therapeutics. Although decitabine is more

chemically stable than azacitidine, it is also degraded into a plethora of products. This is a consequence of hydrolysis and deformylation of the triazine ring and also anomerization of the ribose ring under basic and neutral conditions, which can occur both *in vitro* and *in vivo* (Beisler, 1978). These metabolic changes may potentially generate toxic or mutagenic by-products (Rogstad et al., 2009) and so contribute to the unwanted side effects of this drug. To overcome the instability of these therapeutic agents in aqueous solution, the application of a drug delivery system is an option. Drug delivery systems using a vehicle with hydrophilic-polymeric properties can be fabricated to protect drugs from hydrolysis, increase oral bioavailability, and increase the cellular internalization of the epigenetic drug (Pannier and Shea, 2004). In addition, such modification of demethylating agents allows stable release of the entrapped drug through polymeric diffusion in response to the environment (e.g. ionic strength, pH), which may be effective in allowing oral administration and maintaining prolonged efficacious drug concentrations (Pannier and Shea, 2004).

A variety of natural and synthetic biodegradable materials (polymers) can be used as vehicles for delivery systems of demethylating agents to prevent degradation of the drugs in the acidic environment of the stomach, allowing oral delivery. These polymers can be categorized as either hydrophobic (e.g. poly(lactide-co-glycolide) [PLGA], polyanhydrides), or hydrophilic polymers (e.g. hyaluronic acid [HA], poly(allylamine hydrochloride) [PAH], poly(acrylic acid) [PAA], collagen, and poly(ethylene glycol) [PEG]) (Nair and Laurencin, 2007). PEG is a frequently utilized biopolymer since it is non-toxic, non-immunogenic, and highly soluble in water (Knop et al., 2010). The modification of drugs with PEG chains is an FDA-approved approach. The PEG-drug conjugates impart drugs with the properties of prolonged

residence in the body, a decreased degradation by metabolic enzymes and a reduction or elimination of protein immunogenicity. Because of these favorable properties, PEGylation is now increasing used to enhance the potential of peptides and proteins as therapeutic agents (Veronese and Pasut, 2005). The improvement of the pharmacokinetic index of the modified drug can be measured by detecting the concentration of the uridine compounds in the plasma, liver, and spleen using liquid chromatography-mass spectroscopy.

As yet, only a few demethylating agents have been modified using drug delivery systems. A delivery system for 5-azacytidine was designed by encapsulating this compound in a polymer material (Argemí et al., 2009). The system was established by precipitating two solutions: azacitidine in dimethylsulfoxide (DMSO), and poly-lactic acid (polymer) in methylene chloride. This utilized a supercritical CO_2 antisolvent technique operating in a semi-continuous mode. The precipitated drug-polymer particles were further characterized to determine the percentage of encapsulated drug and establish the delivery kinetics under various release conditions. This system achieved sustained delivery of the drug for several hours and an improved stability of the encapsulated drug, as compared with the pure azacytidine. These results provides proof-of-principle for an approach to enable effective delivery of inherently unstable compounds *in vivo*. The introduction of a drug delivery system can protect the structure from rapid denaturation. The new formulation would allow oral bioavailability, facilitate dosing, reduce administration side effects, and maximize the pharmacologic action of azanucleosides.

1.6.4 Improving cellular uptake

Demethylating agents have limited cellular uptake due to poor bioavailability and a dependency on variably expressed nucleoside transporters (Young et al., 2008). The efficient intracellular accumulation of demethylating cytidine analogs is dependent on the expression of specific transporter proteins such as SLC29A1, SLC29A2, and SLC29A3 which are predominantly located in the plasma membranes in human cells (Young et al., 2008, Baldwin et al., 2005). A low level of intracellular uptake of cytidine analogs limits their incorporation into DNA sequences and therefore reduces their biological activity (Qin et al., 2009).

CP-4200, an elaidic acid (fatty acid), is a derivative of azacitidine, and confers azacitidine with an increased level of nucleoside transportation. CP-4200 has been shown to efficiently cause genome-wide DNA demethylation both *in vitro* and *in vivo* through robust reactivation of epigenetically silenced tumour suppressor genes (Brueckner et al., 2010). When additional elaidic functional groups are modified on the hydroxyl group (CH_2OH) of the 5′ carbon of the ribose ring, the hydrophilicity of CP-4200 is improved, making its uptake less dependent on nucleoside transporters and thus improving its bioavailability (Brueckner et al., 2010). CP-4200 requires activation by plasma membrane lipases, which are present in most cells, suggesting potential clinical application for these drugs in the treatment of solid tumours.

Since lipophilic demethylating agents such as the non-nucleoside agent SG1027 have excellent cellular uptake (Datta et al., 2009), investigation of modification of existing demethylating agents with similar lipophilic moiety is likely to improve their hydrophilicity. Future investigations of the modification of existing

cytidine analogs and non-nucleoside analogs with other potential biodegradable polymer moieties (e.g. elaidic acid, PEG) may further improve cellular uptake of the drugs by improving their hydrophilicity.

1.6.5 Metabolism of cytidine analogs

Demethylating cytidine analogs are prodrugs that are dependent on their phosphorylation and reduction to the active triphosphate-deoxycytidine form for DNA incorporation and to allow DNMT targeting. The conversion of prodrug-cytidine analogs to their active deoxycytidine-triphosphate form requires ribonucleotide reductase (RNR) and cytidine/deoxycytidine kinase (dCK). The activity of RNR is important to regulate the pool sizes of deoxyribonucleotide triphosphates (dNTPs) and to convert ribonucleotide to deoxyribonucleotide. The dCKs are responsible for the phosphorylation of cytidine or cytidine analogs to their triphosphate form, a process that is required for DNA synthesis, especially during S-phase of the cell cycle (Jordan and Reichard, 1998).

Although frequently dividing cells often have high levels of RNR and dCKs (Takeda and Weber, 1981), the presence of physiologic cytidine or other nucleotides could compete for the consumption of available enzymes, resulting in inhibition of phosphorylation of cytidine analog and reduction of the conversion of cytidine analog to their active triphosphate-deoxycytidine form (Bouffard et al., 1993, Takeda and Weber, 1981). On the other hand, a study has also shown that low levels of dCKs in leukemia contribute to the resistance of cells to cytidine analogs due to inefficient conversion of cytidine analogs to their active form for involvement in DNA synthesis (Qin et al., 2009). By transfecting the cells with EGFP (enhanced green fluorescent

protein) -dCK-wild type plasmid, an increased incorporation of decitabine-triphosphate into the DNA was observed in a dCK-deficient leukemia cell line (Qin et al., 2009).

Moreover, to overcome limitations of incomplete conversion of cytidine analogs to their triphosphate form, one approach is to directly employ the active triphosphate-deoxycytidine form of cytidine analogs. The demethylating agent FCDR has been successfully synthesized as the form 5-fluoro-2′-deoxycytidine-5′-triphosphate and used as a substitute for cytidine in DNA synthesis, as shown in DNA polymerase-β assays (Tanaka et al., 1981). However, the effectiveness of these agents is likely to be limited by their poor transport to target tissues, since the human nucleotide-specific membrane transport carriers accept only the dephosphorylated compound (Galmarini et al., 2001). Another possibility is to further investigate the synthesis and usage of non-nucleoside demethylating agents that target the free DNMTs, since these do not require incorporation into DNA (for example RG108, which is a rationally designed DNMT1 inhibitor). RG108 blocks the catalytic site of DNMT1 to impair DNMT1 functionality for methylation without the requirement of DNA incorporation, and reactivation of methylated genes *in vitro* has been observed (see Table 1.2 for more examples) (Mai and Altucci, 2009).

1.6.6 Specific targeting

Apart from limitations such as bioavailability and toxicity, there are possible side effects to non-malignant cells following systemic exposure to demethylating agents (Shichijo et al., 1996). As oncogenes normally remain silenced by DNA methylation in normal tissues, the treatment with demethylating agents may

potentially promote oncogenesis in these tissues by activating these silenced genes (Szyf, 2008, Agrawal et al., 2007). Persistent systemic demethylation may therefore accelerate the initiation and development of these types of cancers, which include lymphomas and sarcomas (Hsieh and Jones, 2003). Global demethylation induced by decitabine has been found to increase invasiveness of the rat chondro-sarcoma cells *in vitro* (Hamm et al., 2009). In mice, reduced methylation following a prolonged systemic demethylating treatment has been shown to cause genomic instability and tumour formation (Gaudet et al., 2003). Adverse effects such as neutropenia, thrombocytopenia, and pneumonia are commonly observed in the patients receiving decitabine treatment (Kantarjian et al., 2006). These adverse effects are considered to be largely due to the nonspecific effects of the drugs (Miyamoto and Ushijima, 2005). Therefore, specific targeting of the demethylating agents to cancer tissues would be important to provide a significant advantage since the exposure of normal tissues would be limited.

NPEOC-decitabine is an analog of decitabine with an N4 protecting modification that confers increased chemical stability and resistance to the acidic conditions associated with oral administration (Byun et al., 2008). NPEOC-decitabine requires activation by human carboxylesterase for biological activity. In the presence of carboxylesterase 1 (CES1), the N4 nitrophenyl group on NPEOC-decitabine is cleaved, resulting in decitabine that can then be incorporated into the DNA sequence. Due to its dependency on metabolism by carboxylesterase for biological activity, NPEOC-decitabine can be specifically used to target cancer cells with high expression of carboxylesterase, while other tissues with low levels of the enzyme will not be affected (Byun et al., 2008, Senter et al., 2001). Unfortunately, high levels of

carboxylesterase are only observed in hepatic carcinomas, thus restricting its potential clinical use to this malignancy. An added limitation of NPEOC-decitabine is its very low solubility in water, which severely limits its bioavailability (Byun et al., 2008).

Among the existing demethylating agents, zebularine has been shown to preferentially target cancer cells without modification (Cheng et al., 2004b). Although zebularine (100 μmol/L) requires a higher dosage than decitabine (1 μmol/L), the lower cytotoxicity of zebularine may overcome this limitation, and it has been shown to elicit minimal side effects during long term treatment *in vivo* (Yoo et al., 2008). In addition to such passive targeting, modification of demethylating agents with cancer cell-specific ligands (e.g. leukemia targeting antibodies) could possibly increase their effectiveness and reduce their nonspecific side effects (Jager et al., 2007).

1.6.7 Development of controlled-release approaches

In addition to the protection of drugs using a drug delivery system, additional modifications to effectively facilitate the controlled release of drugs may further enhance the bioavailability of the drugs. Controlled drug release delivery systems may facilitate biological retention of these demethylating agents, increasing the duration of intra-tumoural exposure of efficacious drug concentrations and minimizing re-methylation of DNA following the removal of treatment. Current examples of modifications that impart a controlled release include hydrogel-cytarabine, PLGA-curcumin, PAA/PAH-cisplatin, and nanogel-gemcitabine. For example, hydrogel is a 3-dimensional hydrophilic network that consists of a framework of insoluble homopolymers or copolymers that allow absorption of >98% of a water-solublized drug for controlled-release application. Hydrogel using PEG as the polymer network

has been FDA approved for the application of tissue adhesion in the US (Reece et al., 2001). The feasibility of the application of hydrogel as a drug vehicle is currently under intensive investigation (Hamidi et al., 2008). The aim of encapsulating drugs in hydrogel is to control the release of the drug by diffusion from the polymer network in response to the physiologic environment. An example of a cytidine analog modified with poly(2-hydroxyethyl methacrylate) hydrogel is the cytotoxic cytidine analog cytarabine (Blanco et al., 1997). The poly(2-hydroxyethyl methacrylate) hydrogel can be varied by cross-linking with varying amounts of polymers or ethylene glycol dimethacrylate, resulting in varying rates of drug release. This is a promising approach, but currently this technology has not been widely applied to the modification of demethylating cytidine analogs.

1.6.8 Nanotechnology and drug delivery

Nanotechnology provides a novel approach to further enhance the delivery of drugs into the target tissues. Nanoparticles are superior as drug delivery systems because the particles are generally <100 nm in diameter, and can be preferentially delivered into diseased tissues as surrounding vasculature is often ‘leaky’ due to abnormal angiogenesis. One example of applied nanotechnology in the delivery of demethylating agents involves the optimization of curcumin, a non-nucleoside demethylating agent, with PLGA using a nanoprecipitation technique (Yallapu et al., 2010). The formulation of curcumin with PLGA allows the drugs to maintain a nanoscale size by the encapsulation into PLGA biodegradable polymer, which avoids particle aggregation. The modified PLGA-curcumin has been shown to enhance cellular internalization. Paclitaxel, doxorubicin, 5-fluorouracil, and dexamethasone

have been successfully formulated using nanomaterials (Suri et al., 2007, Liu et al., 2010).

Nanoparticle-based vehicles can also be utilized to simultaneously package several drugs, providing enhanced therapy (Farokhzad and Langer, 2009). The intravenous administration of the cytotoxic agent gemcitabine-triphosphate encapsulated in nanogel was previously shown to overcome the limiting cellular transportation of phosphorylated cytidine analogs. Using the mouse xenografts of the human breast cancer cell line MCF-7, the gemcitabine-triphosphate nano-particles effectively reduced tumour growth with a lower half maximal inhibitory concentration (IC_{50}) than free gemcitabine (Galmarini et al., 2008). Similarly, cisplatin also functions as a cytotoxic agent by inhibition of DNA repair (Galmarini et al., 2002). Superparamagnetic nanoparticles with layer-by-layer (LBL) surface modification with PAH and PAA have been shown to encapsulate cisplatin and allow controlled release *in vitro* (Thierry et al., 2009).

There has now been proof-of-concept that specific targeting to cancer tissues is possible by use of additional fabrication on the surface of the drug delivery vehicle. This can be achieved by use of complementary functional groups on the polymer, such as antigen-antibody or biotin-avidin, to control internalization of the vehicle into the target cells and controlled release of drug into the desired cellular compartment (Kircheis et al., 2001, Faraasen et al., 2003).

Figure 1.4

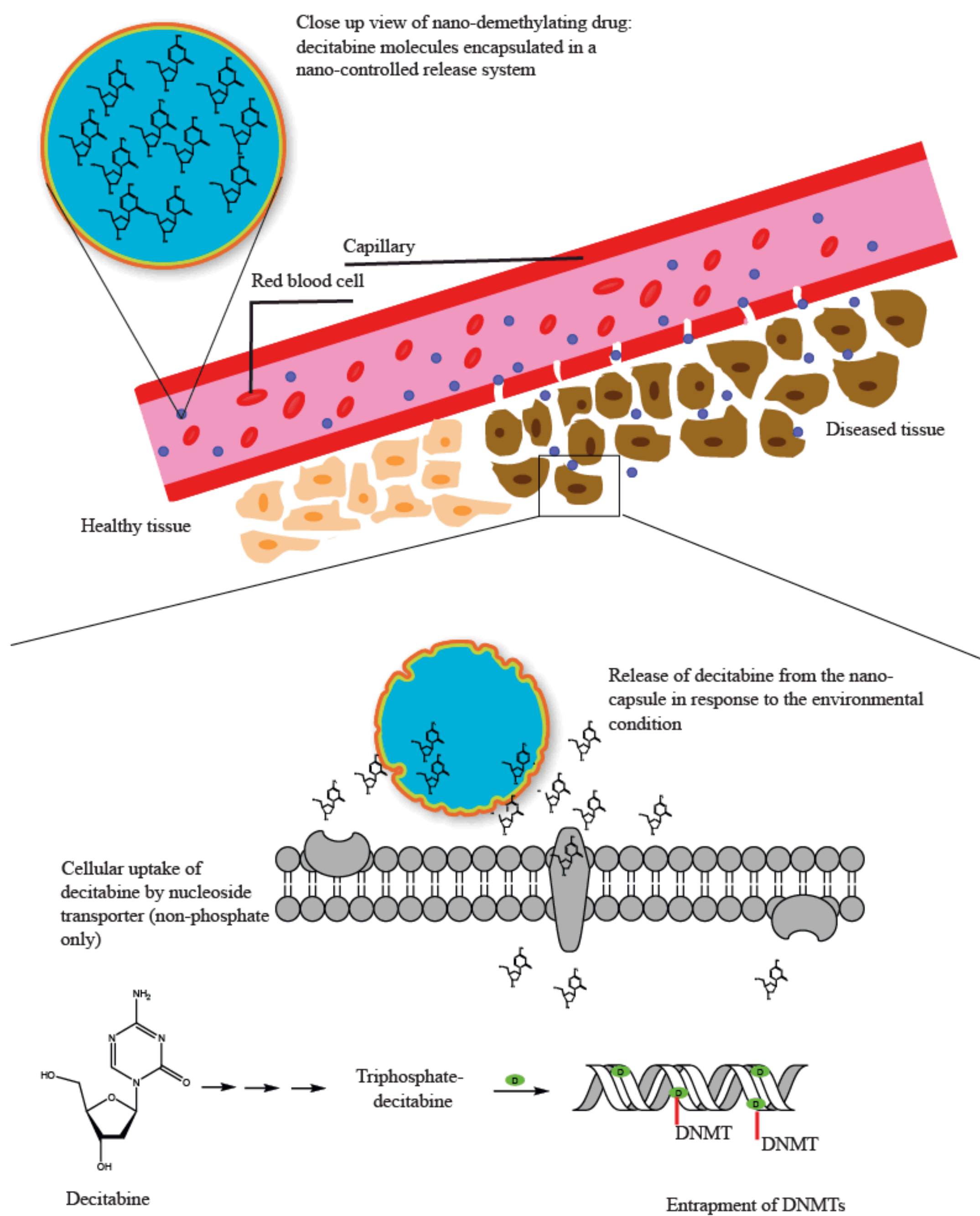

Figure 1.4 Proposed nano-controlled release system of decitabine. Encapsulated decitabine is protected in a nano-controlled release system (e.g. nanogel) and

introduced into the blood stream intravenously, subcutaneously, or orally. These nanoparticles are of the size to be passively released into the diseased tissues through the leaky vasculature surrounding the neoplasm and accumulated intratumorally through the enhanced permeability and retention (EPR) effect. Once the nanodrugs have accumulated in the target tissue, the changes of environmental conditions, such as pH, trigger the release of decitabine from the nanocapsule. Uptake of the released decitabine by the tumour cells occurs through nucleoside membrane transporter proteins. After cellular uptake, decitabine is activated and metabolically converted into its active metabolite (decitabine-5′-triphosphate; D) and is incorporated into DNA during DNA replication whereby it passively inhibits methylation through the entrapment of DNMTs.

1.7 Conclusions

Demethylating agents presently have limited uses as anti-cancer therapies because of the problems of possible adverse effects, limited stability, rapid clearance, inactivation, low bioavailability, and delivery. There are a variety of drug delivery modifications ranging from chemical synthesis of new molecules to modification of existing drugs using nanotechnology that can be exploited to overcome these limitations and allow enhanced efficacy of demethylating agents. There are published examples of approaches that could be utilized to enhance the delivery of demethylating agents. The exploitation of a variety of approaches to further refine and enhance the drug delivery of demethylating agents will enable the wider use of these drugs in cancer therapy. A proposed mode of drug delivery system of decitabine involving nanotechnology and controlled drug release system is shown in Fig. 1.4. A particularly powerful approach is the possibility of encapsulating both demethylating agents and HDAC inhibitors to achieve simultaneous delivery and synergistic effects. These new approaches in drug delivery have the potential to significantly enhance the therapeutic use of demethylating agents.

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CHAPTER 2: DEVELOPMENT OF A NOVEL CELL-BASED ASSAY SYSTEM EPISSAY FOR SCREENING EPIGENETIC DRUGS AND LIPOSOMES FORMULATED DECITABINE

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Development of a novel cell-based assay system EPISSAY for screening epigenetic drugs and liposomes formulated decitabine

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Performed analysis in all samples, interpreted data, wrote manuscript and acted as corresponding author

Certification that the statement of contribution is accurate

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Certification that the statement of contribution is accurate and permission is given for the inclusion of the paper in the thesis

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2.1 Foreword

This chapter is a submitted manuscript with minor modification that reports an assay system, EPISSAY, which enables the detection of compounds with the ability to reverse epigenetic silencing. The EPISSAY is based on a stable cell line with a silenced CMV promoter, that when active, drives a red-fluorescent reporter (RFP) tagged nitroreductase (NTR) transcript. EPISSAY was effective at comparing the epigenetic activity of existing and newly formulated epigenetic drugs. The demethylating agent decitabine is chemically unstable. This drug was encapsulated using a liposome formulation, and characterised by dynamic light scattering and high performance liquid chromatography. The activity of decitabine and encapsulated decitabine were compared using EPISSAY.

Additional supplementary data that are not included in the submitted version of the paper are presented in this thesis. This additional background information regarding development of EPISSAY and the formulation of liposome encapsulated decitabine are included in section 2.8 (Fig. S1 – S7).

2.2 Abstract

Background and Objectives: The epigenetic drug decitabine (a DNA methyltransferase or DNMT inhibitor) is unstable in human plasma as it decomposes by hydrolytic cleavage followed by deamination by cytidine deaminase. To improve the stability of decitabine, the drug was modified using nanotechnology. Despite the potential of improving the delivery of epigenetic drugs, the subsequent assessment of changes in their epigenetic activity is largely dependent on the availability of a suitable and rapid screening bioassay. Here, we describe a cell-based assay system for screening gene reactivation. **Methods:** Decitabine was formulated with PEGylated liposomes to improve its chemical stability, and the size and zeta potential were characterized by dynamic light scattering. A cell-based assay system (EPISSAY) was designed based on a silenced triple-mutated mammalianised nitroreductase B (TMnfsB) fused with Red-Fluorescent Protein (RFP) expressed in the non-malignant human breast cell line MCF10A. EPISSAY was validated using the target gene *TXNIP*, which previously showed a response to epigenetic drugs. **Results:** Following treatment with DNA methyltransferase (DNMT) and histone deacetylase (HDAC) inhibitors such as decitabine and vorinostat, increases in RFP expression were observed, indicating reactivation of *RFP-TMnfsB*. The EPISSAY system was then used to test the potency of decitabine, before and after PEGylated liposomal encapsulation. We observed a 50% higher potency of decitabine when encapsulated in PEGylated liposomes, which is likely to be due to its protection from rapid degradation. **Conclusion**: The EPISSAY bioassay system provides a novel and rapid system to compare the efficiencies of existing and newly formulated drugs that reactivate genes by epigenetic mechanisms.

2.3 Introduction

The prospective of epigenetic-targeted therapy using HDAC inhibitors and DNMT inhibitors has been extensively studied in recent years (Caterina et al., 2010, Hellebrekers et al., 2007). DNA methylation and histone modification are the two major epigenetic mechanisms catalyzed by DNMTs and HDACs, respectively (Richards and Elgin, 2002). HDACs remove the acetyl groups from histones, whilst DNMTs catalyse the transfer of a methyl group from AdoMet to the C5 position of the cytosine pyrimidine ring, both leading to the condensation of chromatin to its inactive state (de Ruijter et al., 2003, Burgers et al., 2002). In cancer cells, an abundance of hypo-acetylated histones is usually associated with DNA hyper-methylation and gene silencing (Ballestar and Esteller, 2002). These findings are the basis for the development of HDAC and DNMT inhibitors as cancer therapeutics. Such compounds block the activity of HDACs and DNMTs, leading to increased expression of epigenetically silenced genes which mediate cellular and metabolic changes such as cell growth arrest, differentiation and apoptosis (Butler et al., 2002, Lyko and Brown, 2005, Gottlicher et al., 2001, Singh et al., 2005, Baylin, 2005).

Hydrophobic vorinostat (suberoylanilide hydroxamic acid, SAHA) and hydrophilic decitabine are FDA approved HDAC and DNMT inhibitors for the treatment of cutaneous T-cell lymphoma and MDS, respectively (Kantarjian et al., 2006, Santini et al., 2007). The combination of vorinostat and decitabine have been shown to have promising activity in patients with MDS without significant toxicity in a phase I clinical trial (Kirschbaum et al., 2009). A liquid chromatography tandem mass spectrometry method has been optimized to simultaneously characterize the pharmacokinetics of vorinostat and decitabine (Patel et al., 2008). The study reported

that vorinostat is more stable than decitabine in human plasma and that the decomposition of decitabine involves two independent mechanisms, hydrolysis followed by deamination by cytidine deaminase.

Under neutral conditions, decitabine has a reported half-life of 7 days at 4°C or 21 hours at 37°C *in vitro* (Stresemann and Lyko, 2008). However, decitabine is degraded more rapidly *in vivo* with a half-life of only 25 minutes (Stresemann and Lyko, 2008). Such chemical instability of decitabine has led to its administration in the clinic as a cold and continuous intravenous infusion in an effort to reach the maximal-tolerated doses required to achieve clinical response (Samlowski et al., 2005, Issa et al., 2004). A patented formulation of decitabine with a cyclodextrin has been shown to improve its stability (Tang, 2006). Modification of decitabine as a dinucleotide, named as S110, protected decitabine against deamination, however, the stability of the triazine ring of decitabine in aqueous solution was not improved following this modification (Yoo et al., 2007).

Herein, we propose to improve the stability of decitabine by modifying the drug using nanotechnology. Liposomes are vesicular structures consisting of hydrated bilayers which self-assemble when a mixture of natural and synthetic phospholipids are dispersed in water. The structure protects the inner compartment from environmental stress, such as pH changes and hydrolysis (Sessa and Weissmann, 1968, Smith et al., 2010). The properties of liposomes allow additional modifications to be achieved with biocompatible polymers such as PEG, which has been shown to prolong blood circulation of certain drugs. For example, FDA approved PEGylated liposomal doxorubicin (DOXIL/Caelyx) for treating ovarian cancer (Rose, 2005). In

the current study, we formulated decitabine with PEGylated liposomes which were ~150 nm and were less toxic compared to the pure drug *in vitro*. The small particle size achieved may allow passive targeting of the drug to the disease tissues by enhanced permeability and retention *in vivo* (Lim et al., 2011).

Despite the potential of improving the delivery of epigenetic drugs, the subsequent assessment of changes in their epigenetic activity is largely dependent on the availability of a suitable and rapid screening bioassay. A commonly used cell-based assay for both DNMT and HDAC inhibitors is the quantification of the re-expression of known epigenetically-silenced genes by quantitative real-time PCR (RT-qPCR) and western blot analysis (Byun et al., 2008, Butler et al., 2002). However, this traditional approach is not high-throughput and may produce gene-specific results. Other assays that have been used include estimation of global DNA methylation using capillary electrophoresis, DNA digestion with methylation-sensitive restriction enzymes, or analysis of specific DNA methylation using bisulfite sequencing and methylation-specific PCR (Villar-Garea et al., 2003). However, these assay systems designated for assaying DNMT or HDAC inhibitors are time-consuming, cumbersome and subject to misinterpretation (Biard et al., 1992, Okochi-Takada et al., 2004, Hassig et al., 2008). Consequently, the rapid identification and validation of novel epigenetic drugs are hampered due to the lack of an efficient screening method.

In this study, a cell-based assay system was developed to screen the epigenetic activity of drugs and liposome-encapsulated decitabine. This assay system is based on mammalian MCF10A cells expressing a fusion protein between RFP and bacterial

nitroreductase (TMnfsB). Human cells expressing TMnfsB are able to metabolize the monofunctional alkylating prodrug 5-(azaridin-1-yl)-2,4-dinitro-benzamide [CB1954] to highly cytotoxic hydroxylamino- and amino-derivatives, which induce rapid cell death (Denny, 2002). The *TMnfsB* open reading frame has been codon optimized to increase the sensitivity of stable human cell lines to the prodrug CB1954 (Grohmann et al., 2009). The condon optimised *TMnfsB* had less non-specific cytotoxicity and was 40-80 fold more sensitive to CB1954 than the wild-type *nfsB* when expressed in mammalian cells. An epigenetic assay system was developed by identifying clones where gene expression of RFP-TMnfsB was suppressed at the transcriptional level, but could be restored by subsequent treatment with epigenetic drugs. Since RFP expression in these clones is low, reactivation of gene expression can be detected by flow cytometry. The liposome-encapsulated decitabine was found to have higher potency than the pure drug using this novel assay system.

2.4 Materials and Methods

2.4.1 Plasmids

The *TMnfsB* was generated by subcloning the nitroreductase open reading frame from existing constructs kindly provided by Grohmann et al. (Grohmann et al., 2009) into the pDsRED-C1-monomer vector at a *Xho*I/*Bam*HI site. A retroviral plasmid pLNCX2-*RFP-TMnfsB* expressing RFP-TMnfsB fusion was generated by subcloning the *RFP-TMnfsB* coding fragment from the existing construct pDsRED-*TMnfsB* (*Sna*BI/*Bam*HI) into the pLNCX2 vector (*Sna*BI/*Bgl*II). All constructs were confirmed by sequencing using appropriate primers (Table 2.1).

Table 2.1 PCR primers used in this study

Target gene	Forward primer (5'—3')	Reverse primer (5'—3')	Product size (bp)	Annealing temperature (°C)
TXNIP [NM_006472.3]	GCCCGAGCCAGCCAACTCAA	ACCCGAAGGCTCTTGCCACG	379	58
RFP-TMnfsB	CACACACACACACATGATCAACCATGGACAACACCGAGGACGTCATCA	TTCCTGTGCATGTCGGCGGT	1100	58
TMnfsB	AAGAGCGCCGCTGGCAACTAC	TTCCTGTGCATGTCGGCGGT	206	58
β-actin [NM_001101.3]	TACCTTCAACTCCATCATGAAGTG	CCGGACTCGTCATACTCCTGCTTG	267	57

TXNIP: Homo sapiens thioredoxin interacting protein

RFP-TMnfsB: exogenous red-fluorescent protein and triple-mutated nitroreductase gene B

TMnfsB: exogenous triple-mutated nitroreductase gene B

β-actin: Homo sapiens actin, beta (ACTB)

2.4.2 Cell culture

All human cell lines were purchased from the American Type Culture Collection (ATCC) except the Phoenix retrovirus producer cell line which was kindly provided by Prof. Garry Nolan of Stanford University (United States). All cell lines were grown in the ATCC recommended media.

2.4.3 Reagents

CB1954 (soluble to 2 mg/mL in aqueous solution), decitabine (soluble to 50 mg/mL in aqueous solution), 2(1H)-pyrimidinone riboside (zebularine; soluble to 16 mg/mL in DMSO) and RG108 (soluble to 10 mg/mL in DMSO) were purchased from Sigma. RG108 is known as an ineffective DNMT inhibitor (Lim et al., 2011) and was used as a negative control. Vorinostat (10 mM) was kindly supplied by Dr. Lisa Butler of The University of Adelaide (South Australia). All drugs were dissolved in DMSO except decitabine, which was prepared in water for liposomal formulation. The synthetic lipids 1,2-dioleoyl-sn-glycero-3-[phospho-rac-(1-glycerol)] sodium salt (DOPG), 1,2 distearoyl-sn-glycero-3-phosphocholine (DSPC), 1,2-distearoyl-sn-glycero-3-phosphoethanolamine-N-[amino(polyethylene glycol)-2000] ammonium salt (DSPE-PEG2000) and natural cholesterol lipid were purchased from Avanti Polar Lipids.

2.4.4 Preparation of liposomal decitabine

Liposomal formulations were prepared according to the method developed by Sunoqrot and colleagues with minor modifications (Sunoqrot et al., 2011). Briefly, 5

mg (32.5 mol%) DOPG, 4.9 mg (32.1 mol%) DSPC, 1.8 mg (3.3 mol%) DSPE-PEG2000 and 2.4 mg (32.1 mol%) cholesterol were dissolved in 5 mL of chloroform. Thin lipid films were generated after removing the solvent in a rotary evaporator for 2 hours at room temperature. Liposomes were formed when thin lipid films (4 mM) were hydrated in 5 mL of water or 0.88 mM decitabine dissolved in water for 1 hour at room temperature and stored at 4°C. The samples were extruded ten times using 200 and 400 nm polycarbonate membranes to obtain unilamellar liposomes.

2.4.5 Liposomes characterisation

The size and zeta potential of liposomes were characterized by dynamic laser light scattering (Malvern Zetasizer Nanoseries). Data are expressed as the mean plus standard deviation of three technical repetitive measurements. For determination of encapsulation efficiency, free decitabine in the supernatant was collected after centrifugation at 82,508 xg for 30 minutes at 4°C and measured by high performance liquid chromatography (HPLC, Shimadzu LC-10AT) using a XTerraTM C8 analytical column at 254 nm. Milli-Q water was used as the mobile phase with a flow rate of 0.8 mL/min. The limit of quantification of decitabine is 10 ng/mL (Lin et al., 1981). The encapsulation efficacy of decitabine was defined as the mass ratio between the amount of drugs incorporated in liposomes and that used in the liposome preparation.

2.4.6 Generation of stable cell line and clonal selection

Recombinant retrovirus encoding RFP-TMnfsB was produced using the Phoenix packaging cell line transfected with Lipofectamine 2000 (Invitrogen) according to the recommended protocol. Stable cell lines expressing RFP-TMnfsB

were generated by G418 selection of MCF10A cells transduced with retrovirus expressing RFP-TMnfsB (Fig. S2). G418-resistant MCF10A cells were grown into colonies in 10 cm dishes and potential clones where TMnfsB was spontaneously silenced were isolated by treating these colonies with 5 μM of CB1954 for 72 hours. Surviving colonies, which were potentially epigenetically silenced, were isolated as CB1954-resistant clones. The integrity of *RFP-TMnfsB* in CB1954-resistant clones was determined by screening using RT-PCR. Finally, colonies with epigenetically silenced *RFP-TMnfsB* insert were identified by assessing TMnfsB and RFP expression using RT-PCR and flow cytometry, respectively, after treatment with epigenetic drugs.

2.4.7 Quantitative real-time polymerase chain reaction (RT-qPCR)

RNA and DNA from the cells were extracted using the RNeasy plant mini kit (Qiagen) and the DNeasy Blood and Tissue Kit (Qiagen), respectively. cDNA was generated using random primers and 20 U of reverse transcriptase (Promega). *TXNIP*, *TMnfsB* and *RFP-TMnfsB* expression were determined by RT-qPCR using IQ^{TM} SYBR green supermix (Biorad) and primers listed in Table 2.1. Cycling conditions were: 10 min at 95°C followed by 40 repeats of 95°C for 10 s, annealing at appropriate temperature for 15 s and extension at 72°C for 10 s. β-actin expression was used for normalisation of target gene expression.

2.4.8 Western blotting

Western blot analysis of RFP-TMnfsB fusion protein expressed in HEK293T cells was performed using a rabbit polyclonal anti-RFP antibody (Invitrogen) or

mouse anti-β-actin antibody (Sigma-Aldrich), and a secondary donkey anti-rabbit IgG-HRP (GE Healthcare) or a sheep anti-mouse IgG-HRP (GE Healthcare) (Kumar et al., 2008). Total cellular proteins were extracted as described previously (Kumar et al., 2006) and visualized by an Enhanced Chemiluminescence Detection Kit (Amersham Biosciences).

2.4.9 Flow cytometry

The reactivation of epigenetically silenced RFP-TMnfsB was determined by flow cytometry. Cells were plated at 40% 24 hours prior to treatment. The approximate doubling time of the cells observed by light microscopy is 48 hours. Cells were treated with each drug (decitabine 1, 5, 10, 30 and 50 μM; zebularine 50, 100, 250 and 500 μM; RG108 10 and 100 μM; vorinostat 1 and 2 μM) for 48 or 72 hours in triplicate. The red-fluorescence of cells was analyzed at a log scale of geometric mean of FL3-H using FACSCalibur flow cytometer (BD). Data were processed using WinMDI v2.8 software.

2.4.10 Statistical analysis

Data were analyzed by GraphPad Prism (GraphPad Software, Inc.) using unpaired two-tailed t-tests, and linear and nonlinear regression.

2.5 Results

2.5.1 Development of a cell-based assay system EPISSAY for screening epigenetic drugs

The triple-mutated mammalianized version of *nfsB*, *TMnfsB* (Grohmann et al., 2009), was selected for developing the assay system as it showed the highest sensitivity to the lethal effect of CB1954 (Fig. S1). The schematic of the development of cell-based assay system for gene reactivation and the chemical structures of epigenetic drugs used in this study are presented in Fig. 2.1. A stable MCF10A clone (T1) was generated which expressed the cytomegalovirus (CMV) promoter driven RFP-TMnfsB fusion by growing in media containing G418 for over two months. Expression of the RFP-TMnfsB fusion protein was confirmed by western blot analysis (Fig. S2). The CMV promoter is known to be gradually silenced over a period of months in culture and can be reactivated by subsequent treatment with epigenetic drugs (Choi et al., 2005, Meilinger et al., 2009). The clone T1 showed increased expression of RFP-TMnfsB fusion protein after treatment with DNMT inhibitors (decitabine and zebularine) by western blot and flow cytometry analyses (Fig. 2.2A). We observed that this was not due to auto-fluorescence of basal MCF10A cells (Fig. 2.2B). This confirmed that clone T1 contained cells in which *RFP-TMnfsB* was silenced, possibly by epigenetic mechanisms.

To identify the optimum clone for the basis of the assay system, cells of the T1 clone were treated with CB1954 to kill RFP positive cells which were expressing RFP-TMnfsB. The surviving clones were those where the CMV promoter was epigenetically silenced. The CB1954-resistant clones showed different levels of red-

fluorescence (Fig. 2.2C). Whereas the clones LT1, LT2 and LT3 had significantly lower levels of basal red-fluorescence (relative red-fluorescence= ~8), clones HT1, HT2, HT3, and HT4 showed a high level of red-fluorescence (relative red-fluorescence= ~50). Despite differences in the base levels of red-fluorescence, the red-fluorescent signals of all clones increased after treatment with decitabine and zebularine (Fig. 2.2C). Among the six clones, LT1 showed the highest sensitivity to treatment with DNMT inhibitors.

Figure 2.1

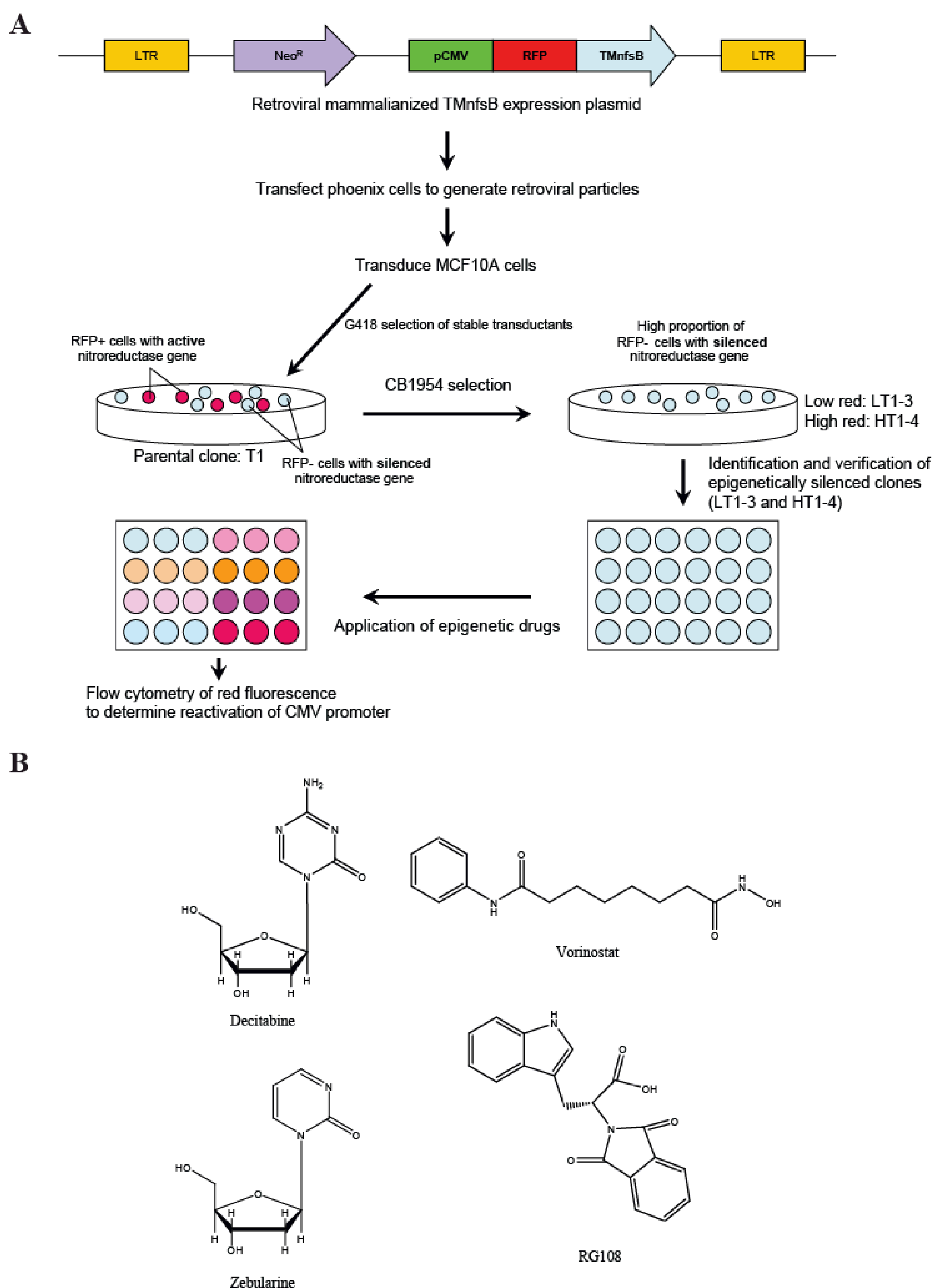

Figure 2.1 The design of cell-based assay system. (A) Schematic showing different steps in development of the cell-based assay system for testing efficiency of epigenetic drugs. (B) Chemical structure of the epigenetic drugs used in this study.

Figure 2.2

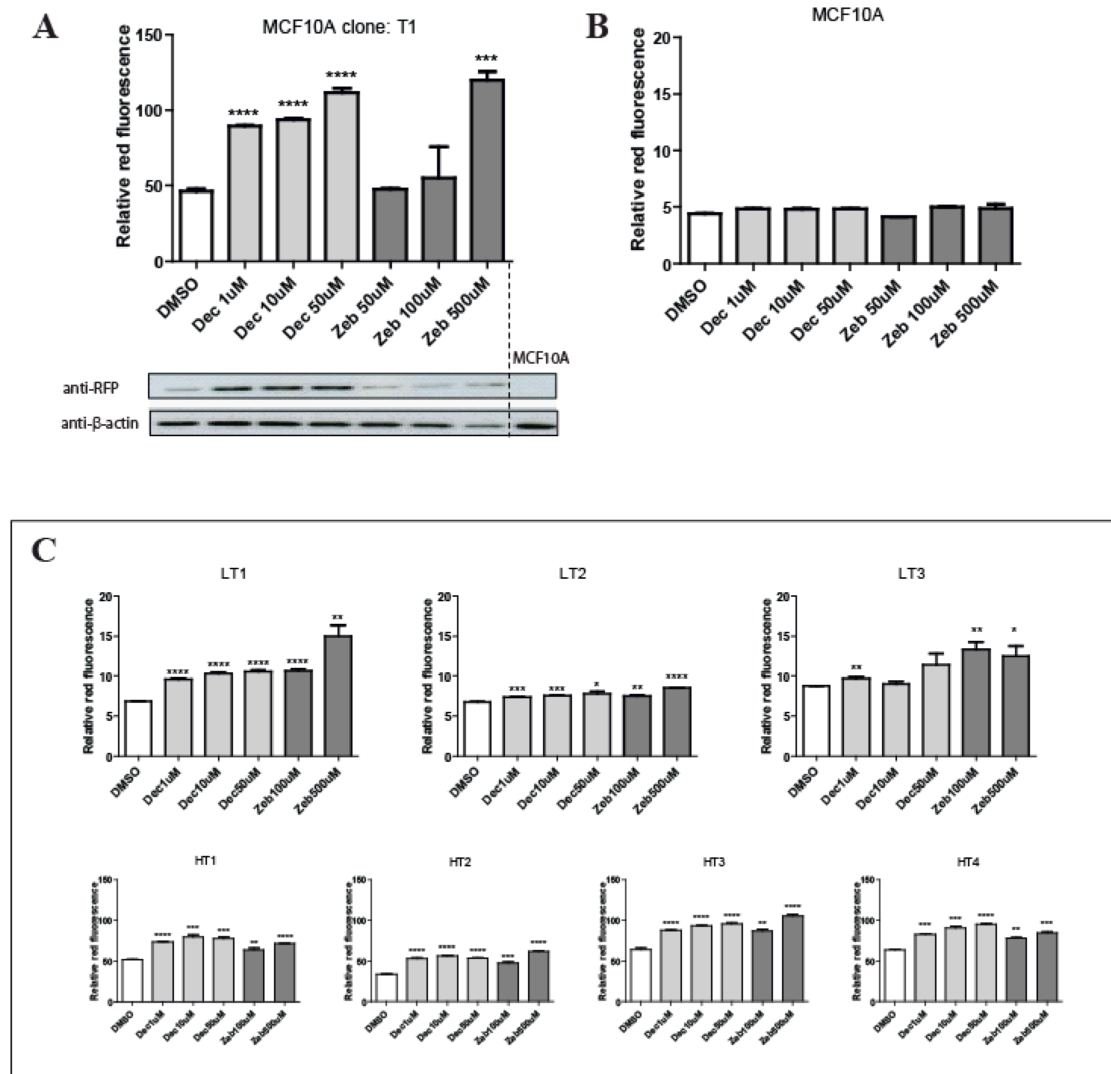

Figure 2.2 Flow cytometric assessment and western blot of the parental (A) *RFP-TMnfsB* expressing clone T1 and (B) untransduced MCF10A cells. The size of RFP-TMnfsB protein is 52 kDa. (C) Flow cytometric assessment of the CB1954-resistant clones generated from T1. Top panel: low fluorescent clones LT1, LT2 and LT3. Bottom panel: high fluorescent clones HT1, HT2, HT3 and HT4. Treatments were: decitabine 1, 10, 50 μM or zebularine 50, 100, 500 μM for 72 hours in triplicate in <1% v/v DMSO. Red-fluorescent reading is the gated geometric mean value of FL3-H. Note the different y axis scales for each panel. Unpaired two-tailed t-test, data

expressed as mean ± SEM. * = $p < 0.05$, ** = $p < 0.01$, *** = $p < 0.001$, **** = $p < 0.0001$.

2.5.2 Proof of principle of the assay system

To further validate the approach, three CB1954-resistant clones LT1, LT3 and HT3 were selected and treated with decitabine and/or vorinostat for 72 hours, with media changes every 24 hours to maintain drug levels. An increased level of red-fluorescence was observed after treatment in all three clones (Fig. 2.3A).

Since the red-fluorescent signal should reflect expression of the *RFP-TMnfsB* gene, levels of *TMnfsB* mRNA were quantified in the treated cells (Fig. 2.3B). There was a significant correlation between levels of red-fluorescence and *TMnfsB* expression in the LT1, LT3 and HT3 clones treated with decitabine and/or vorinostat ($p< 0.0001$), confirming that the red-fluorescent signal is directly related to the levels of *TMnfsB* message.

To further validate these findings, the reactivation of an independent endogenous target gene was also assayed. The gene chosen was *TXNIP* (thioredoxin interacting protein), which was previously shown to be reactivated after treatment with decitabine and/or vorinostat (Ahsan et al., 2006, Butler et al., 2002). The amount of *TXNIP* in the LT1, LT3 and HT3 cells was assessed after treatment with decitabine and/or vorinostat. A linear relationship of red-fluorescence and *TXNIP* mRNA expression was observed ($p= 0.0023$) (Fig. 2.3C). Taken together, our data clearly showed that increases in the levels of red-fluorescence signal are correlated with the endogenous *TXNIP* reactivation in the cells treated with epigenetic drugs, suggesting that this cell-based assay, which we have named EPISSAY, can be used for screening the activity of epigenetic drugs.

Figure 2.3

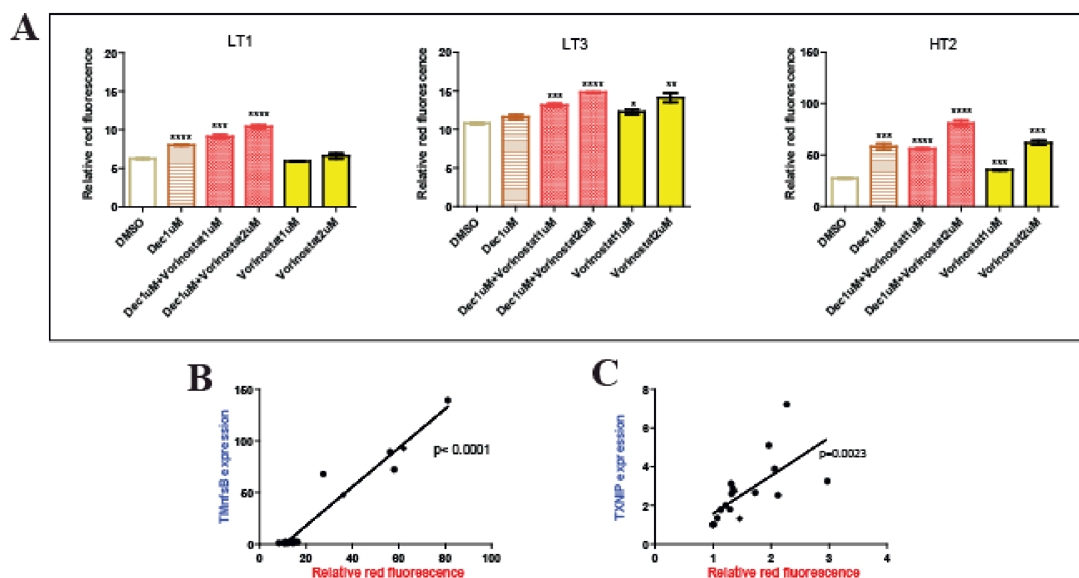

Figure 2.3 Proof of principle of the assay system. (A) Flow cytometric assessment of CB1954-resistant clone expressing *RFP-TMnfsB*. HT2, LT1, and LT3 were treated with 1 µM decitabine and/ or 1, 2 µM of vorinostat (SAHA) for 48 hours. The average red-fluorescence of the treated cells (n=3) were correlated with the mRNA expression of (B) *TMnfsB* of treated HT2 and LT3; (C) *TXNIP* of treated LT1 and LT2 (n=1) cells normalised to *β-actin* expression. The red-fluorescent reading for *TXNIP* analysis was normalised to vehicle control. All treatments contain <1% v/v of DMSO.

2.5.3 The physiochemical properties of liposomal decitabine

Decitabine is an unstable compound that undergoes hydrolysis (Lin et al., 1981) and degradation by cytidine deaminase (Chabot et al., 1983). To improve the stability of decitabine, we formulated decitabine loaded liposomes by thin-film hydration as multilamellar liposomes with a broad size distribution of 871 ± 69 nm (Table 2.2). A narrow size distribution of decitabine-loaded liposomes was obtained by extruding the suspension through 400 nm and 200 nm filters to achieve a size of 138 ± 5 nm as unilamellar liposomes. The polydispersity index (PDI) of these extruded liposomes was less than 0.5 of the scale of 1 and liposomal formulation achieved an encapsulation efficacy of 55.1 ± 3.4% (0.15 μg of decitabine/mg of lipid). The zeta potential of decitabine-loaded liposomes before extrusion was similar to the empty liposomes. The zeta potential of decitabine-loaded liposomes before extrusion -69.9 ± 2.8 increased to -40.2 ± 4.3 mV after extrusion. Overall the physiochemical data confirmed the decitabine-loaded liposomes are highly dispersed and achieved a smaller size <150 nm after extrusion. The potency of these newly formulated decitabine-loaded liposomes was subsequently compared with the free drug using the EPISSAY system.

Table 2.2 The physiochemical properties of the liposomes

Sample	Mean diameter, nm (± SD)	PDI	Zeta potential, mV (± SD)
Decitabine-loaded liposomes (MLVs)	871 ± 69	0.358	-69.9 ± 2.8
Decitabine-loaded liposomes (*E)	138 ± 5	0.296	-40.2 ± 4.3
Drug-free Liposomes (MLVs)	1070 ± 77	0.744	-60.6 ± 2.7
Drug-free liposomes (*E)	146 ± 1.6	0.137	-56.8 ± 0.9

*E: unilamellar liposomes extruded using 200 and 400 nm polycarbonate membranes.

MLVs: multilamellar vesicles

PDI: polydispersity index

2.5.4 The potency of liposomal formulated decitabine and pure drug tested in the EPISSAY system

To compare the potency of a panel of epigenetic drugs and newly formulated decitabine, LT1 cells were treated with these drugs for 72 hours, with or without a media change with fresh drug every 24 hours. Continuous treatment is often required as genes can be re-methylated after the removal of decitabine (Si et al., 2010). With a media change, 2 μM vorinostat and unilamellar decitabine-loaded liposomes at 30 μM were found to be more potent than pure decitabine and zebularine (Fig. 2.4A). Notably, we observed a linear dose-dependent response in cells treated with unilamellar decitabine-loaded liposomes from 5 to 30 μM. There is a 50% increase of potency of the unilamellar decitabine-loaded liposomes compared with pure decitabine at 30 μM. Cells treated with 1 μM decitabine in the presence, or absence, of 2 μM vorinostat gave similar results.

To investigate whether liposomal formulation protects decitabine from degradation, LT1 cells were treated with different concentrations of decitabine and liposomal decitabine for 72 hours without a media change (Fig. 2.4B). The potency of multilamellar decitabine-loaded liposomes and pure decitabine without media change were lower than those with the media change. Nevertheless, the potency of unilamellar decitabine-loaded liposomes (10 μM) was maintained. Although unilamellar decitabine-loaded liposomes (30 μM) have the highest potency without media change, this was slightly reduced in comparison with replacing the drug every 24 hours. Taken together, our data showed that the potency of decitabine is improved when delivered as a unilamellar liposomal formulation.

Figure 2.4

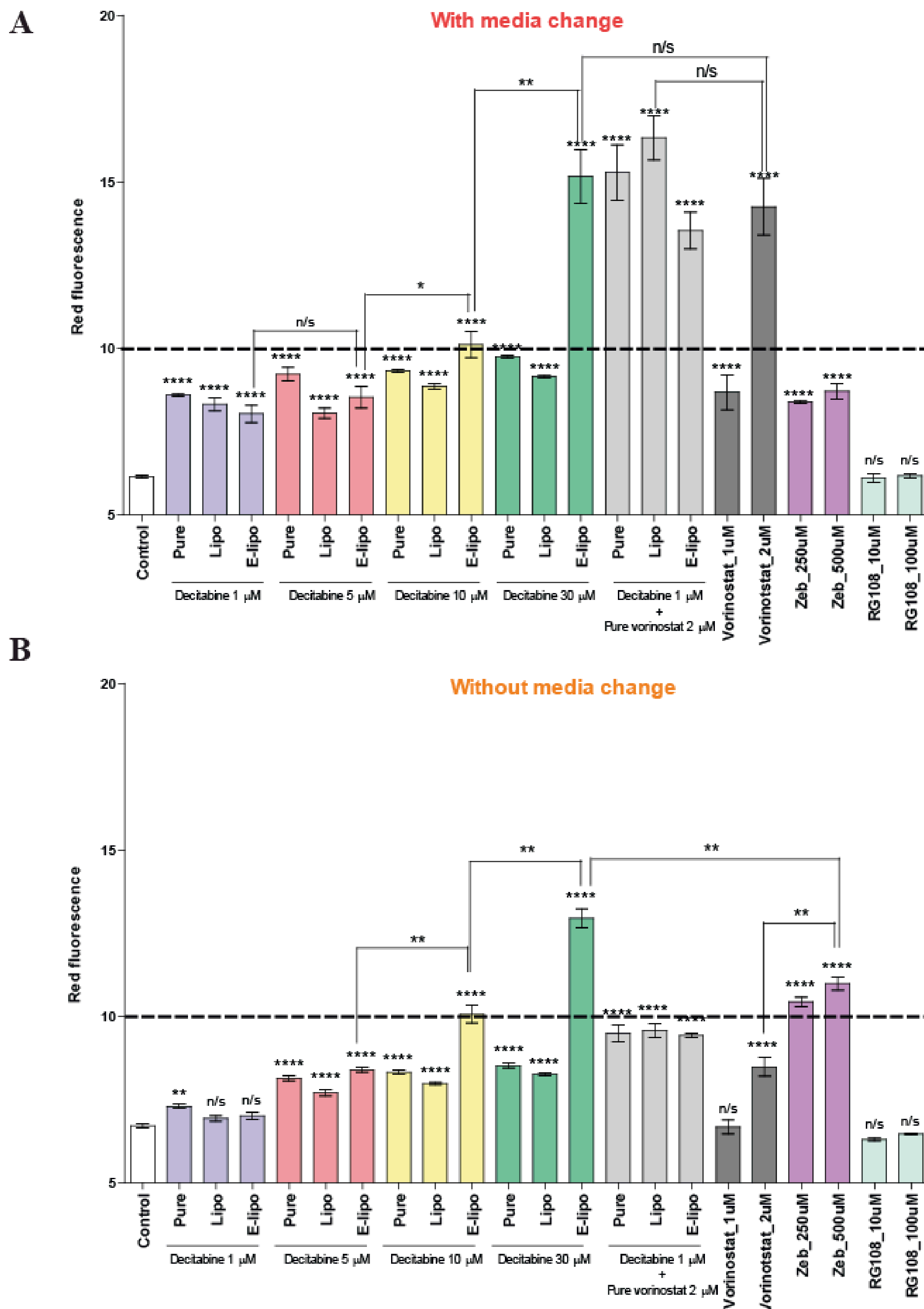

Figure 2.4 The comparison of pure and newly-formulated epigenetic drugs using EPISSAY. Flow cytometric assessment of LT1 cells treated with epigenetic drugs.

Treatments were: liposomal formulated or pure decitabine 1, 5, 10, 30 μM and/ or pure vorinostat 1, 2 μM; pure zebularine 250, 500 μM; pure RG108 10, 100 μM (A) with or (B) without media change for 72 hours in triplicate. The gated geometric mean values of FL3-H (red-fluorescence) were normalised to the vehicle control, drug-free liposomes and water. Lipo: multilamellar decitabine-loaded liposomes; E-lipo: unilamellar decitabine-loaded liposomes. Pure: drug without modification. Unpaired two-tailed t-test, data expressed as mean ± SEM. * = $p < 0.05$, ** = $p < 0.01$, *** = $p < 0.001$, **** = $p < 0.0001$.

2.6 Discussion

EPISSAY, a cell-based assay system for screening of epigenetic drugs was developed based on the human non-malignant breast epithelial cell line MCF10A expressing the well-characterized CMV promoter driving RFP fused with a mammalianized version of the bacterial nitroreductase (*NTR*) gene. The *NTR* gene encodes an oxygen-insensitive flavin mononucleotide-dependent enzyme that generates nicotinamide adenine (phosphate) oxidase (NAD(P)H), and has been used in gene-directed enzyme prodrug therapy (Mitchell and Minchin, 2008). Since treatment of mammalian cells expressing bacterial *NTRs* with CB1954 results in its chemical reduction to two cytotoxic metabolites, exposure of the derivative of MCF10A with CB1954 was used as a strategy for the selection of cell lines with silenced *NTR* genes from a mixed population. For these studies, *TMnfsB* with a mammalianized coding sequence (Grohmann et al., 2009) was selected as the candidate *NTR* gene as cells expressing *TMnfsB* showed the highest sensitivity towards CB1954 treatment. In stable clones expressing *RFP-TMnfsB*, treatment with CB1954 resulted in greater 90% cell death (*data not shown*).

The integrity of the stably-integrated gene in the selected clones was confirmed by sequencing the PCR amplified DNA. In addition, these clones were verified by treatment with the known epigenetic drugs decitabine, zebularine or vorinostat; all of which resulted in increased red-fluorescence due to reactivation of the CMV promoter. Nevertheless, there was a linear relationship between *TMnfsB* expression and the red-fluorescent signal confirming that levels of gene message and translated protein are directly related. Epigenetic response was further confirmed by measuring expression levels of a known independent endogenous gene *TXNIP*.

EPISSAY is a rapid multi-well formatted assay for screening compounds with epigenetic activity. Standard methodologies used to assess epigenetic compounds are based on quantitative real-time PCR and western blot analysis of genes known to be epigenetically silenced in a particular cell line. For example, quantification of the re-expression of a endogenous gene p16 in human T24 bladder carcinoma cell line was previously used (Cheng et al., 2003). Such approaches are time-consuming as they require cell collection for RNA and protein extractions prior to analysis. Other cell-based assay systems which use exogenous expression of genes (e.g. *Escherichia coli* β-D-galactosidase gene with and green fluorescent reporter) have previously been investigated for their potential in screening epigenetic drugs by using fluorescent microscopy and plate readers. However, these other systems have limitations such as the non-quantitative data obtained and/ or additional sample treatments required (e.g Paraformaldehyde fixing, the addition fluorogenic compounds) prior to screening (Byun et al., 2008, Villar-Garea et al., 2003, Biard et al., 1992). EPISSAY requires limited sample preparation, can be formatted for multi-well plates, and rapid results can be generated from RFP reading using flow cytometry to obtain quantitative data.

Decitabine is a demethylating agent that is FDA approved as an anti-cancer agent (Stresemann and Lyko, 2008). Since decitabine is degraded *in vivo* with a half-life of only 25 minutes, daily treatments are required to maintain appropriate drug levels both *in vitro* and *in vivo* (Hollenbach et al., 2010). To improve the stability and effectiveness of decitabine, the drug was encapsulated in PEGylated liposomes, as liposomes are known to protect drugs from degradation, allow controlled release of drug and have a low cytotoxicity (Fig. S6 and S7) (Immordino et al., 2006). This formulation achieved an encapsulation efficiency of ~50%. Only 3.3 mol% of PEG

2000 was used in this study as a higher PEG content is known to reduce adsorption of liposomes onto cells (Er et al., 2009). This low PEG coverage was confirmed by the retention of negative zeta potential.

The liposomes generated after hydration of thin lipid films with water or decitabine had a broad size distribution of around 0.8 to 1 µm in diameter, i.e. multilamellar vesicles (MLVs). Limitations of MLVs include the heterogeneity of size distribution, multiple internal compartments, limited cellular uptake and inconsistencies among different preparations (Barbara and Michael, 2006). To overcome these problems, liposomes were extruded through filters with defined pore size (200 nm and 400 nm) to obtain unilamellar liposomes. Although extrusion does not affect the encapsulation efficiency (Colletier et al., 2002), it narrowed the size distribution of the liposomes from 1 µm to approximately 150 nm. The smaller size of the drug-loaded liposomes has been reported to passively targeting disease tissues due to their enhanced angiogenesis (Maruyama et al., 1999).

We used of EPISSAY system to compare the potency of decitabine, decitabine-loaded liposomes, combination of decitabine/vorinostat, vorinostat and zebularine. EPISSAY cells were treated for 72 hours with indicated epigenetic drugs with or without media change. Following a media change every 24 hours to maintain the drug level, the potency of pure decitabine and zebularine were similar. However, the potency of zebularine was higher in the absence of media changes as compared to pure decitabine, which is a likely reflection of the higher stability of zebularine and consistent with the previous findings (Zhou et al., 2002). In addition, decitabine encapsulated in unilamellar liposomes showed 50% more potency than pure

decitabine, suggesting that decitabine was protected in the liposomes and slowly released into the media. These results were supported by a controlled release study comparing the drug release of decitabine from unilamellar and multilamellar liposomes (Fig. S6). This showed that the release rate of decitabine from unilamellar liposomes was slower, suggesting unilamellar liposomal formulation may decrease the rate of degradation of decitabine by providing protection to the drug. Low-dose decitabine is known to have a long-term effect in recovering cell proliferation control whereas high-dose decitabine has a immediate cytotoxic effect (Tsai et al., 2012). In addition, the liposomal formulation and the presence of phospholipids in the cell media could also contribute to the enhancement of decitabine activity (McAllister et al., 1999, Egbaria and Weiner, 1990). Taken together, this liposomal formulation is likely to achieve a low-dose and an effective treatment to the cancer cells.

Collectively, the liposomal decitabine that was synthesised here was validated as a more potent epigenetic drug. However, we have only confirmed this *in vitro*. An *in vivo* study of liposomal decitabine is recommended to assess its applicability for clinical use, and to confirm if the present limitations of decitabine use in the clinic could be overcome by this formulation. The use of liposomes/PEG to encapsulate drugs to improve their bioavailability and stability is now gaining momentum with a number of drugs eg doxorubicin (Rose, 2005), rhenium radionuclides (Hrycushko et al., 2011) and dexamethasone phosphate (Anderson et al., 2010), liposome-encapsulated doxorubicin now having FDA approval.

In this study, we have constructed and evaluated a novel bioassay for epigenetic compounds. The readout of the EPISSAY system is red-fluorescence,

allowing adaptation to a multi-well format allowing high throughput, rapid, and cheap bioassay. In this pilot study, EPISSAY was successful in providing evaluation of different liposomal formulations of decitabine. The EPISSAY can detect the gene reactivating effects of decitabine or zebularine. Using SEQUENOM MassARRAY EpiTYPER, no major changes in methylation of the CMV promoter was detected in the EPISSAY cells before and after treatments with decitabine (Fig. S3-5). Although vorinostat is known as a HDAC inhibitor to activate gene expression, zebularine and decitabine are usually considered to function as demethylating agents or DNMT inhibitors (Daskalakis et al., 2010). However, there are now multiple studies that show these agents can also function as HDAC inhibitors (Halaban et al., 2009, Scott et al., 2007, Radhakrishnan et al., 2008, Savickiene et al., 2012, Lavelle et al., 2006). This suggests that the *TMnfsB* gene was most likely silenced by histone modification rather than direct methylation of the CMV promoter. This is a potential of adopting this assay as a high throughput, rapid and low cost epigenetic drug screening platform are unique aspects of the EPISSAY system. We conclude that our EPISSAY bioassay system provides a novel and rapid system to screen the efficiencies of epigenetic and newly formulated drugs. We also suggest that this liposomal formulation could be useful in efficiently delivering therapeutic agents that are normally unstable.

2.7 Acknowledgements

Assistance with confocal microscopy was kindly provided by Ghafar Sarvestani (IMVS, Adelaide).

2.8 Supplementary Information

This supplementary information provides more detailed Materials and Methods and Results that could not be included in the manuscript due to the requirements of the journal to which the paper was submitted. Included is information concerning the selection of the most sensitive version of the nitroreductase gene (*TMnfsB*), validation of the fusion RFP-TMnfsB protein in stable clones after G418 selection, methylation analysis of the CMV promoter in the EPISSAY cells, and controlled release and toxicity studies of the liposomes formulated decitabine.

A) Supplementary Materials and Methods

i) Plasmids

In order to identify the most appropriate version of the NTR gene in the human context, plasmids expressing a variety of bacterial NTRs were generated. The bacterial *nfsA* gene was amplified by polymerase chain reaction (PCR) using primers (forward 5′ - CACACACACACACACACACTCGAGCCATGACGCCAACCATTGAACTTATTTG- 3′ and reverse 5′ - CACACACACAGGATCCTTAGCGCGTCGCCCAACCCTGTTTG- 3′) from *E. coli* plasmid and cloned into the multiple cloning site of pDsRED-C1-monomer vector. The other *NTRs*, pDsRED-*nfsB* (wild type *nfsB*), pDsRED-*MnfsB* (mammalianised *nfsB*) and pDsRED-*TMnfsB* expression constructs were generated by subcloning these *NTR* open reading frames from existing constructs kindly provided by Walther's group (Grohmann et al., 2009), into the pDsRED-C1-monomer vector at the XhoI/ BamHI site (Fig. S1).

ii) Transfection

HEK293T cells were plated at 1.5×10^5 cells/ well of a 12-well plate 24 hours prior to transfection. HEK293T cells were transiently transfected with the four different *NTR* open reading frames in the pDsRED-C1-monomer vector using Lipofectamine 2000 (Invitrogen) following the manufacturer's protocol, and cultivated in the presence of various concentration of CB1954 for 24 hours (Fig. S1).

iii) Confocal microscopy

Cells were visualized using a 10× objective on a Nikon C1 laser scanning confocal microscope equipped with epifluorescence optics (Fig. S1).

iv) Methylation analysis of the CMV promoter

CMV promoter is responsible for driving the transcription of RFP-TMnfsB in the cell-based assay system. To analyse the methylation level of the CMV promoter, DNA samples were bisulphite-treated and purified using the Epitect Kit (Qiagen) according to the manufacturer's protocol (Fig. S3-5). Unconverted genomic DNA was included as a negative control. *In vitro* transcription and uracil-specific cleavage of the amplified products were undertaken before analysis of the samples by matrix-assisted laser desorption and ionisation time-of-flight mass spectrometry (MALDI-TOF-MS; SEQUENOM EpiTYPER Platform) as previously described (Wong et al., 2008). Two overlapping regions (CMV_1 and CMV_2) of the bisulfite-treated CMV promoter sequence were amplified using the PyroMark Kit (Qiagen) and primers given below. Lowercase denotes sequence tags added to facilitate downstream EpiTYPER analysis.

CMV_1:

5′-AGGAAGAGAGAATAGTAATTAATTACGGGGTTATTAGTTTATAGTTT-3′

5′-CAGTAATACGACTCACTATAGGGAGAAGGCccataaaatcatatactaaacataata-3′

CMV_2:

5′-AGGAAGAGAGATTTTTTTATTTGGTAGTATATTTACGTATTAGTT-3′

5′-CAGTAATACGACTCACTATAGGGAGAAGGCttcactaaaccaactctacttatataa-3′

v) Controlled release study of liposomes formulated decitabine

A controlled release study was performed using dialysis tubing (regenerated cellulose tubing, Mw cut-off 12000, 43 mm flat width, Crown Scientific, Australia) incubated in phosphate buffered saline (PBS) at 37°C. A 0.25 mL decitabine liposome suspension was added to the dialysis tubing immersed in a beaker with 10 mL of PBS as the release medium. Aliquots of 0.1 mL were collected from the solution outside the dialysis tubing at different time points. The volume of PBS was maintained by addition of 0.1 mL PBS after each withdrawal. The concentration of decitabine in each sample was determined using HPLC (Fig. S6).

vi) Cell viability assay

The toxicity of liposomal formulation was tested using cell viability assay. The cells were treated similarly as described in section 2.4.9. After treatment with liposomal decitabine, trypsinised cells were stained with 2 μg/mL 7-amino-actinomycin-D solution (7AAD, Invitrogen) for 10 minutes at room temperature as described before (Pishas et al., 2011). Data were processed using FACSCalibur flow cytometer (BD) and WinMDI v2.8 software (Fig. S7).

B) Supplementary Results

Figure S1

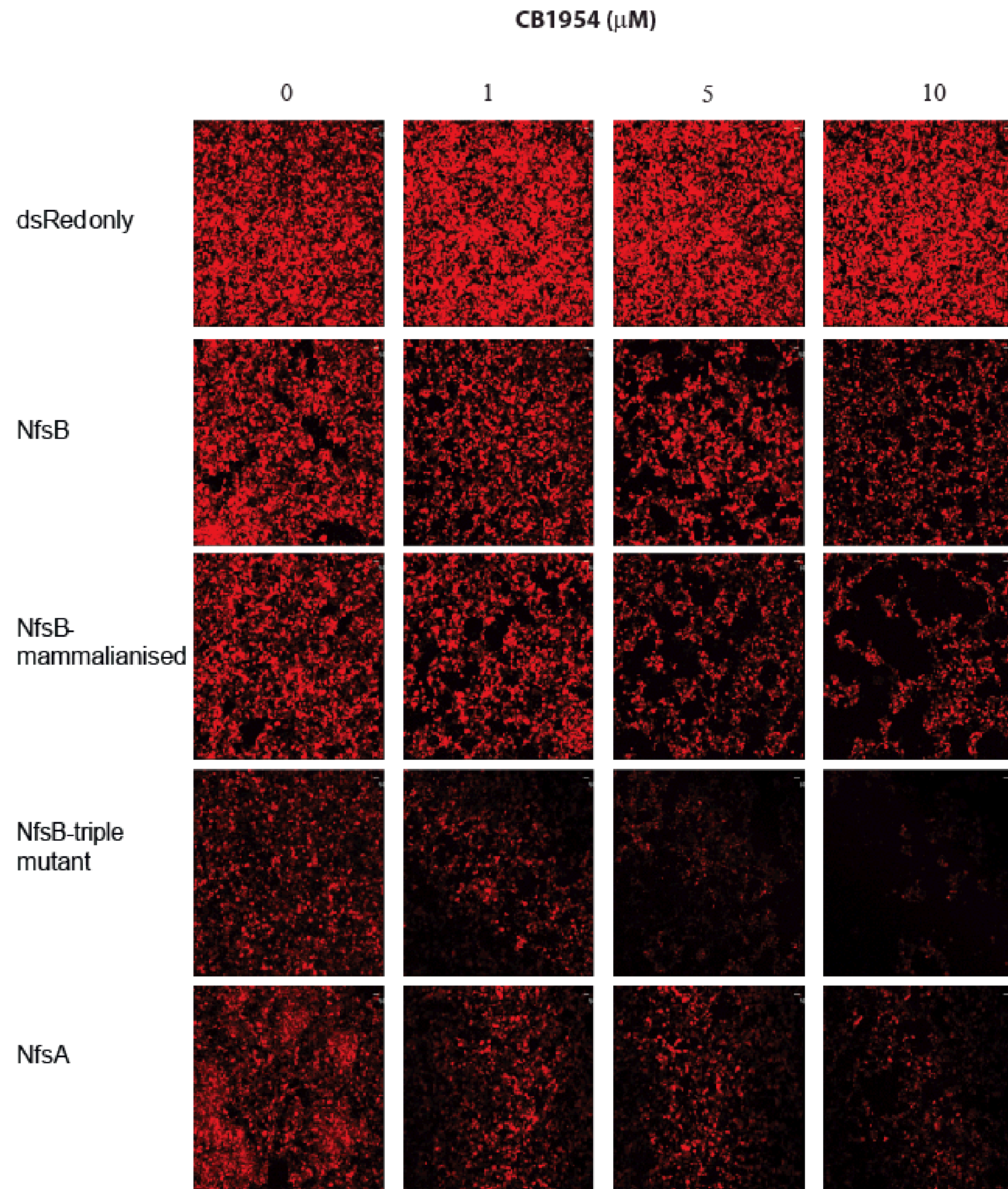

Figure S1 Sensitivity of different nitroreductase genes to CB1954. Transiently transfected HEK293T cells with (A) pDsRED-monomer-C1 vector, (B) pDsRED-

nfsA, (C) pDsRED-nfsB, (D) pDsRED-MnfsB, (E) pDsRED-TMnfsB and incubated with 0, 1, 5, 10 µM of CB1954 for 24 hours at 37°C/ 5% CO_2. All contain 0.2% v/v DMSO. The decrease in red-fluorescence indicates cell death.

Results: *The NTR genes include bacterial nitroreductase gene A (nfsA), bacterial nitroreductase gene B (nfsB), mammalianised nitroreductase gene B (MnfsB) and triple-mutated mammalianised nitroreductase gene B (TMnfsB). To select the most sensitive version of the NTR gene, the percentage of cell death following CB1954 treatment was compared in transiently transfected HEK293T cells expressing different bacterial NTR genes, wild-type nfsA, wild-type nfsB, mammalianised nfsB and triple-mutated mammalianised nfsB.*

There was a trend of increased cell death in NTR expressing cells with increasing concentration of CB1954 (Fig. S1). The cells without NTR showed no cell death, confirming the selectivity of the treatment towards cells expressing NTR. Cells expressing the mammalianised version of nfsB have a higher sensitivity to CB1954 than either nfsA or nfsB as they showed the highest levels of cell death at the lower concentration of CB1954. Among them, cells expressing the triple-mutated mammalianised version of nfsB, TMnfsB, possessed the highest sensitivity toward CB1954 and was selected as the basis for the assay system. [See section 2.5.1 for more information]

Figure S2

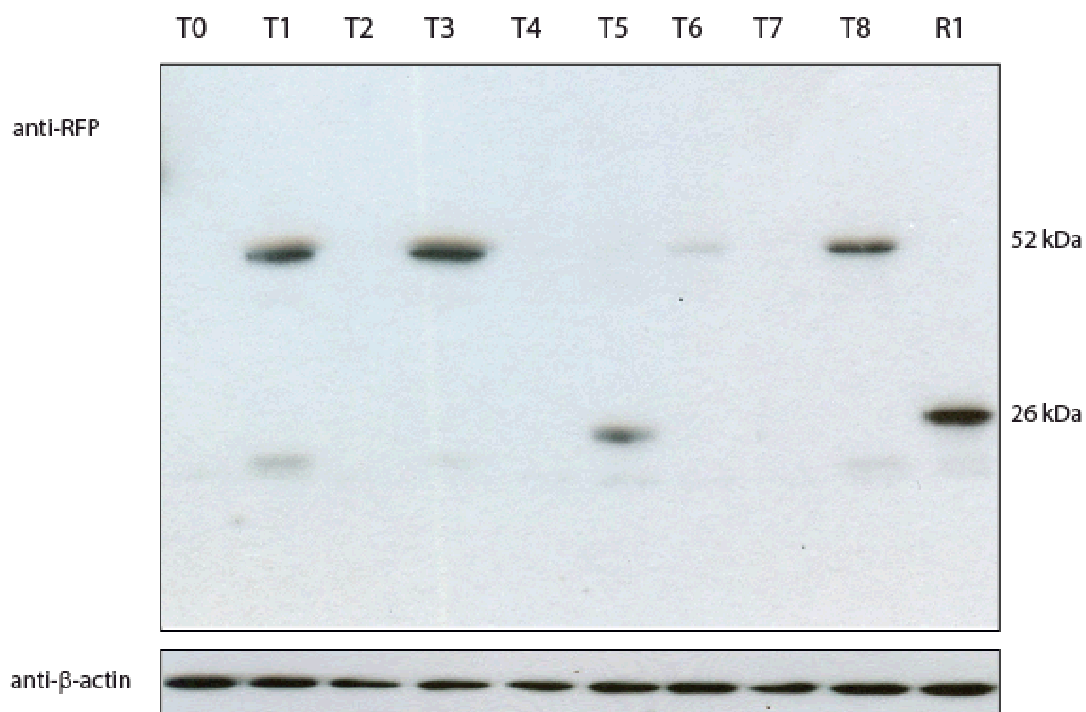

Figure S2 Western blot of the single clones of stable MCF10A cells transduced with viruses expressing RFP-TMnfsB (T0 to T8) 52 kDa and *RFP* only (R1) 26 kDa using anti-RFP antibody.

Results: *The method to generate stable cells expressing RFP-TMnfsB was recorded in section 2.4.6. In this western blot, four clones T1, T3, T6 and T8 showed an intact fusion protein of RFP-TMnfsB (Fig. S2). T1 was selected as the parental clone for downstream experiment as mentioned in section 2.5.1.*

Figure S3

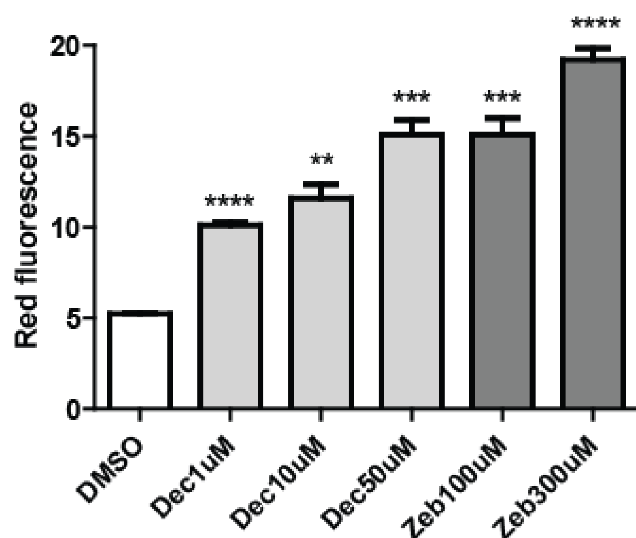

Figure S3 Flow cytometric assessment of EPISSAY cells prior to CMV methylation analysis (Fig. S4). Cells were treated with decitabine 1, 10, 50 μM or zebularine 50, 100, 300 μM for 72 hours in triplicate in <1% v/v DMSO. Red-fluorescent reading was the gated geometric mean value of FL3-H. Unpaired two-tailed t-test, data expressed as mean ± SEM. ** = p< 0.01, *** = p< 0.001, **** = p< 0.0001.

***Results**: EPISSAY cells were treated with decitabine and zebularine, and assayed by flow cytometry. There was an increase in red-fluorescence resulting from reactivation of the CMV driven RFP-TMnfsB gene (Fig. S3). This increase in gene expression maybe due to changes in methylation of the CMV promoter. This was tested by methylation analysis, Fig. S4.*

Figure S4

Figure S4 Epigram showing methylation levels of the CMV promoter generated from SEQUENOM EpiTYPER Platform (see Supplementary Materials and Methods iv).

This epigram showed % CpG methylation of CMV promoter in overlapping regions of CMV_1 and CMV_2 amplicons of RFP-TMnfsB expressing clones treated with epigenetic drugs are indicated (n=2). Dec: decitabine; Zeb: zebularine. LT1 is the CB1954-resistant clone, which subsequently in used as the basis of EPISSAY. T1 is the parental clone without CB1954 selection and has a higher red-fluorescent background than LT1. The CpG units are as defined in Fig. S5.

***Results**: The levels of red-fluorescence of LT1 (EPISSAY) cells were previously shown to increase following treatment with either zebularine or decitabine (Fig. S3), suggesting possible demethylation of the CMV promoter in LT1 cells. Cells from treatments with demethylating agents were analysed for expression of RFP-TMnfsB (Fig. S3) and for CMV promoter methylation (Fig. S4). The T1 (parental clone) and LT1 did not demonstrate consistent differences in methylation across the CMV promoter. The samples analysed following treatments with demethylating agents showed inconsistent levels of methylation at some sites. These data suggest gene silencing in these cells was not due to DNA methylation (Fig. S4).*

Figure S5

CMV_1

```
                                                                      Unit 2
                                                         CMV start site (2374 bp)
                              Unit 1
CGTAATAGTAATTAATT|ACGGGGTT|ATTAGTTTATAGTTTATATATGG|AGTTTCGCGTT
                 Unit 3        Unit 4            Unit 5     Unit 6
|ATATAATTT|ACGGT|AA|ATGGTTCGTTTGGTTG|ATCGTTT|A|ACG|ATTT
Unit 7       Unit 8        Unit 9                   Unit 10
TCGTTT|ATTG|ACGTT|AATAATG|ACGT|ATGTTTTTATAGTA|ACGTT|AAT
                              Unit 11                     Unit 12
AGGGATTTTTTATTG|ACGTT|AATGGGTGGAGTATTT|ACGGT|AAATTGTT
                                                      Unit 13
TATTTGGTAGTATATTAAGTGTATTATATGTTAAGT|ACGTTTTTT|ATTG|A
Unit 14       Unit 15        Unit 16                                          2631
CGTT|AATG|ACGGT|AA|ATGGTTCGTTTGGT|ATTATGTTTAGTATATGATTTTATGG
```

CMV_2

```
2630
                                     Unit 1          Unit 2                  Unit 3
GATTTTTTTATTTGGTAGTATATTT|ACGT|ATTAGTT|ATCGTT|ATTATTATGGTG|ATGC
                                       Unit 4       Unit 5         Unit 6
GGTTTTGGT|AGTATATTA|ATGGGCGTGG|AT|AGCGGTTTG|ATTT|ACGGG
                                          Unit 7
G|ATTTTTAAGTTTTTATTTTATTG|ACGTT|AATGGGAGTTTGTTTTGGTAT
               Unit 8                Unit 9          Unit 10
TAAAATTA|ACGGG|ATTTTTTAAA|ATGTCGT|AATA|ATTTCGTTTT|ATTG|
Unit 11       Unit 12     Unit 13     Unit 14
ACGT|AA|ATGGGCGGT|AGGCGTGT|ACGGTGGG|AGGTTTATATAAGTAGAGTT
                         CMV ends (2906)
GGTTTAGTGAATCGTTAGATTCGTTAGCGTTATCG
```

Figure S5 Amplicon design and the target region for methylation analysis. Bisulfite treated sequence of CMV promoter regions: CMV_1; CMV_2. [T bold: cytosine from non-CG converted to T; *italic smaller font*: primer target sequence; all CGs: bold; CG underlined: analysed CGs; |Unit|: fragment with different mass and size generated by enzymatic base specific cleavage.] [See Fig. S4]

Figure S6

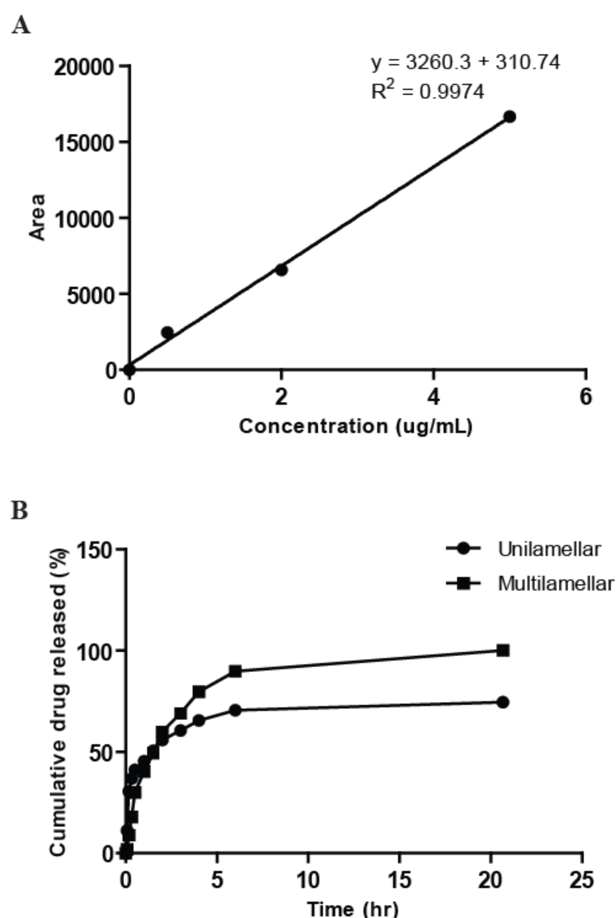

Figure S6 Controlled release study of liposomal decitabine. (A) The standard plot of pure decitabine produced using HPLC at 254 nm (retention time = 6.554 ± 0.003 minutes). (B) Drug release profiles of unilamellar and multilamellar liposomal decitabine at different time intervals generated using the standard plot of pure decitabine.

***Results**: In Fig. S6 50% of decitabine was released from both unilamellar and multilamellar liposomes at ~90 minutes. At 4 hours, the rate of release of decitabine from unilamellar (65%) was slower than multilamellar liposomes (80%). This observation supports the potency of unilamellar liposomal decitabine that we previously observed in Fig. 2.4. [See section 2.6]*

Figure S7

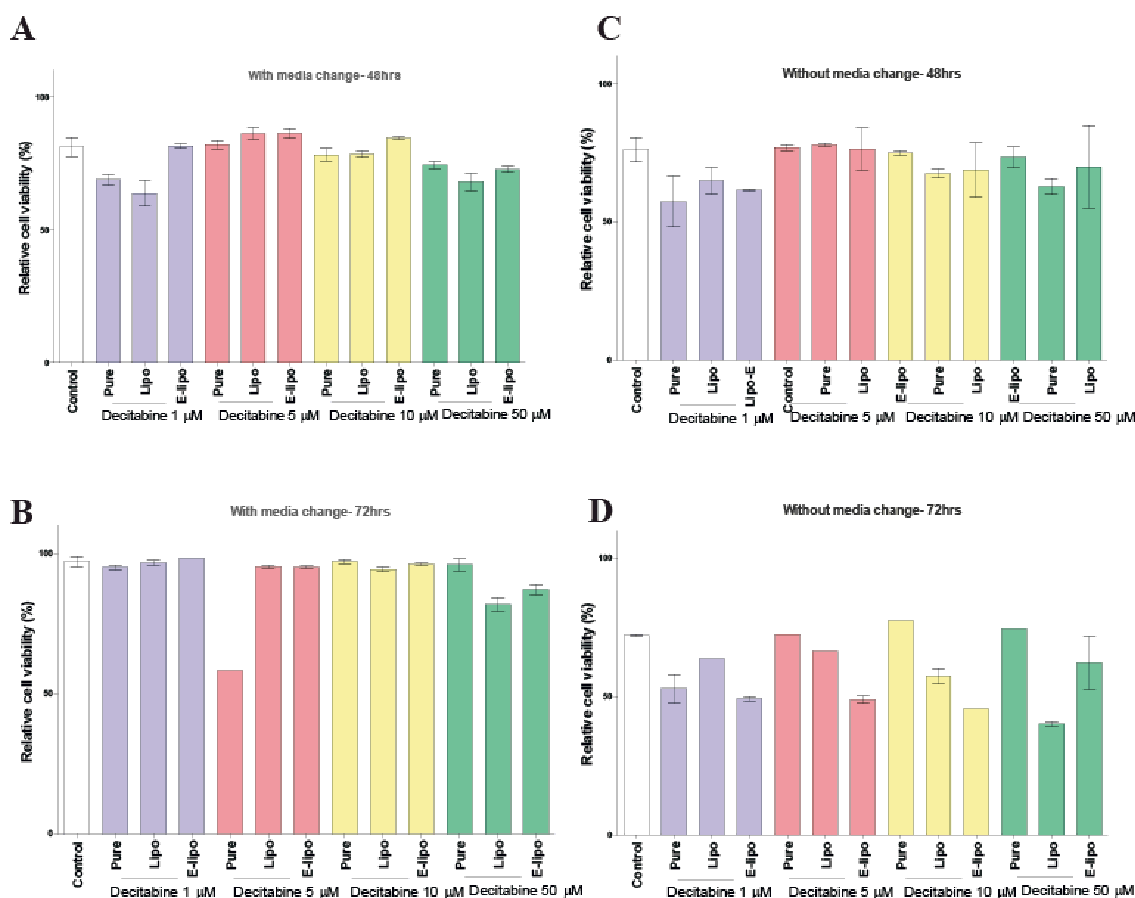

Figure S7 The comparison of the toxicity of pure and newly-formulated decitabine using EPISSAY. EPISSAY cells were treated with 1, 5, 10, 50 μM of liposomal formulated or pure decitabine with (A) or without (B) media change for 48 hours; or with (C) or without (D) media change for 72 hours in triplicate. The % viable cells was normalised to the vehicle control, water, 72 hours with media change. Lipo: multilamellar decitabine-loaded liposomes; E-lipo: unilamellar decitabine-loaded liposomes. Pure: drug without modification. Unpaired two-tailed t-test, data expressed as mean ± SEM.

***Results**: This study tested the cytotoxicity of the liposomal formulation by comparing the percentage of viable cells in EPISSAY system after treatment with liposomal decitabine and pure decitabine (Fig. S7). With media change, no significant difference between liposomes formulated decitabine and pure decitabine was found. Without media change, the relative viable cells of liposomal decitabine and pure decitabine were lower than those with the media change. Overall, no significant difference between pure and liposomes formulated decitabine was observed, suggesting this liposomal formulation has minimal cytotoxicity in vitro. [See section 2.6]*

C) Supplementary Discussion

In this additional study, we have identified the most sensitive version of nitroreductase gene, *TMnfsB*, in the human context and validated the protein expression of RFP-TMnfsB in the stable clones. DNA methylation studies of the CMV promoter of the silenced *RFP-TMnfsB* fusion gene in the clone used for the EPISSAY system did not detect any significant levels of DNA methylation as compared with the parental clone which express higher level of *RFP-TMnfsB*. Using the EPISSAY system, liposome formulated decitabine shows increased potency compared with the pure drug or zebularine (Fig. 2.4). Controlled release and toxicity studies confirmed that this liposomal formulation has a low toxicity *in vitro* and has improved the stability of decitabine. Further *in vivo* studies are required to explore the clinical potential. The implications of these findings are discussed in section 2.6.

CHAPTER 3: SPECIFIC-SITE METHYLATION OF TUMOUR SUPPRESSOR ANKRD11 IN BREAST CANCER

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3.1 Foreword

The remainder of this thesis is an exploration of the role of epigenetic silencing of the gene *ANKRD11* in breast cancer. Previously our laboratory has characterised the large 296 kDa protein ANKRD11 (Neilsen et al., 2008). This novel protein contains an ankyrin repeat domain which interacts with, and is a co-activator of, p53 (Neilsen et al., 2008). The *ANKRD11* gene is in a region of the long-arm of chromosome 16 (16q24.3) that is frequently involved in loss-of-heterozygosity in breast cancer (Powell et al., 2002). Preliminary expression data showed *ANKRD11* has a low expression in breast cancer.

In the following studies we determined the expression of *ANKRD11* in breast cancer cell lines and clinical samples. We then investigated in these samples if *ANKRD11* was silenced by DNA methylation. This manuscript has recently been published in *European Journal of Cancer*. These studies are further continued in Chapter 4, which investigates the possible the relationship between *ANKRD11* expression and levels of the *GLI1* transcription factor.

3.2 Abstract

ANKRD11 is a putative tumour suppressor gene in breast cancer, which has been shown in our laboratory to be a co-activator of p53. Our data suggest that downregulation of *ANKRD11* is associated with breast tumorigenesis. Treatment with epigenetic drugs can reactivate gene expression by DNA demethylation, histone modification and/or activation of cellular stress pathway (Halaban et al., 2009, Scott et al., 2007, Radhakrishnan et al., 2008, Savickiene et al., 2012, Lavelle et al., 2006, Sutton et al., 2002). Our study showed that breast cancer cell lines treated with DNA demethylating agents resulted in up-regulation of *ANKRD11* expression. One of the gene silencing mechanisms, promoter DNA methylation, was investigated for its responsibility in downregulating *ANKRD11* expression. The transcriptional activity of a CpG-rich region 2 kb upstream of the transcription initiation site of *ANKRD11* was investigated using Dual-luciferase reporter assays. The constructs carrying -661 to -571 bp promoter sequence showed significant transcriptional activity. Using the SEQUENOM EpiTYPER Platform, the region between -770 to +399 bp was analysed in 25 breast tumours, four normal breast tissues and five normal blood samples. The region between –770 to -323 bp was shown to be frequently methylated in breast tumours. The methylation patterns of all analysed CpGs in this region were identical in the normal and tumour samples, except for a 19 bp region containing three CpG sites. These sites had significantly higher levels of methylation in tumours (40%) compared to normal samples (6%). Our findings support the role of *ANKRD11* as a tumour suppressor gene and suggest that aberrant DNA methylation of three CpGs in a 19 bp region within the *ANKRD11* promoter may be responsible for its downregulation in breast cancer.

3.3 Introduction

Breast cancer is one of the most common cancers in women, and in 2008, it accounted for 6% of cancer-related deaths worldwide (Jemal et al., 2011). Cancer is driven by activation of oncogenes and inactivation of tumour suppressor genes. Inactivation of tumour suppressor genes results from mutation, loss of heterozygosity (LOH) and/or epigenetic silencing (Addou-Klouche et al., 2010).

ANKRD11/ ANCO-1 is a putative breast cancer suppressor and is a co-activator of p53 (Neilsen et al., 2008). ANKRD11 is also shown to interact with p160 nuclear receptor co-activators and inhibits ligand-dependent transcriptional activation (Zhang et al., 2004). Recent reports that *ANKRD11* is a candidate gene for autism and neurocognitive impairments in patients with 16q24.3 microdeletion syndrome also suggest its normal role in development (Isrie et al., 2011, Willemsen et al., 2010).

ANKRD11 is located at 16q24.3, a predominant region of loss of heterozygosity (LOH) in breast cancer (Callen et al., 2002). LOH is a common mechanism for the loss of normal function of one allele of a gene. The other copy can be inactivated by an epigenetic mechanism, such as mutation or DNA methylation, resulting in reduced *ANKRD11* expression. DNA methylation is catalysed by DNMTs, where the recruitment of methyl-binding proteins and HDACs results in the formation of transcriptionally repressive chromatin states (Katoh et al., 2006). Typically, gene transcription is silenced by hyper-methylation of the CpG-rich promoter region, which is usually located in the 5′ untranslated region (5′ UTR) or the 5′ exon/ intron region of a gene (Brenet et al., 2011, Harder et al., 2010).

Herein, we determined the expression of *ANKRD11* in both normal and breast cancer tissues and investigated the role of CpG methylation of the promoter of *ANKRD11* in modulating its expression. Our results suggest that specific CpG methylation of *ANKRD11* promoter is associated with breast cancer.

3.4 Materials and Methods

3.4.1 Clinical sample collections

Tissues were obtained with informed consent from 30 breast cancer patients and five normal breast reduction mammoplasties performed at the Royal Adelaide Hospital between 2003 and 2011. Formalin fixed paraffin-embedded tissue was used for immunohistochemistry. Unfixed tissues were stored in RNA*later* solution (Ambion) at -20°C and subsequently used for DNA and RNA extraction using the Allprep DNA/RNA mini kit (Qiagen). Relevant clinical data was retrieved from patient's records including human epidermal growth factor receptor 2 (HER2), estrogen receptor (ER), progesterone receptor (PR), and proliferation index (MIB-1) status. Genomic DNA extracted from five normal blood samples was kindly supplied by Dr. Kathryn Friend of the Women's and Children's Hospital (Adelaide).

3.4.2 Cell culture

All human breast cell lines were purchased from the ATCC. MCF-7 and ZR75-1 cells were maintained in RPMI supplemented with 10 μg/mL of insulin (Invitrogen) and 1mM of sodium pyruvate. MDA-MB-231 and MDA-MB-468 cells were cultured in Dulbecco's modified Eagle's medium (DMEM). Media were supplemented with 10% fetal bovine serum and 100 U/mL penicillin-streptomycin-glutamine (Invitrogen). MCF-10A cells were cultured in DMEM nutrient mixture F12 HAM with 20 ng/mL EGF, 0.5 μg/mL of hydrocortisone, 100ng/mL of cholera toxin, 10μg/mL of insulin and 5% horse serum (Invitrogen). All reagents were purchased from Sigma unless specified. Cells were grown at 37°C with 5% CO_2.

3.4.3 Treatment with DNA methyltransferase inhibitors

24 hours prior to treatment, cells were plated at 1×10^5 cells/ well of a 6-well plate. Treatments consisted of decitabine (Sigma) or zebularine (Sigma) for 72 hours. Drug levels were maintained by replacing media containing the relevant concentration of drug every 24 hours. Following completion of the experiment, cells were harvested and RNA and DNA extracted using the RNeasy plant mini kit (Qiagen) and the DNeasy Blood and tissue kit (Qiagen), respectively.

3.4.4 Luciferase reporter system

3.4.4.1 *ANKRD11* promoter activity and site-directed mutagenesis

Six different regions of the human *ANKRD11* gene including 5′ UTR and exon 1 (-2000 to +306) were cloned into a promoterless luciferase reporter vector, pGL3-Basic (Promega, Madison, WI), designated as P1 (-2000 to +306), P2 (-2000 to -661), P3 (-571 to +306), P4 (-862 to +107), P5 (-689 to +306) and P6 (-689 to -543) (Fig. 3.4 and Fig. 3.9). Specific mutations in the P6 construct (Fig. 3.9B) were generated by overlap PCR using primers listed in Table 3.1. All constructs were verified by DNA sequencing.

3.4.4.2 In vitro methylation of *ANKRD11* promoter

Recombinant HpaII, M.SssI and HhaI methylases (New England Biolabs) were used to methylate specific sites of the pGL3-P6 construct. Plasmid DNA (4µg) was treated with 4 units of the specific methylase and 640 µM of S-adenosylmethionine in the manufacturer’s recommended buffer at 37°C for 4 hours,

incubated at 65°C for 20 minutes to inactivate the enzymes and then purified using QIAquick PCR kit (Qiagen). Methylation was confirmed by the lack of endonuclease restriction site by HpaII for HpaII methylation; BstUI for M.SssI methylation; and HinP1I for HhaI methylation.

3.4.4.3 Transient transfection and luciferase assay

Briefly, 1×10^5 of MCF-10A cells were plated/ well of 24-well tissue culture dishes. Transfections were performed with lipofectamine 2000 reagent (Invitrogen) following the manufacturer's protocol. An internal control plasmid for *Renilla* luciferase expression, pRL-TK (Promega), was included in each transfection. 24 h post-transfection, cells were lysed and the Firefly and *Renilla* luciferase activities were sequentially measured using the Dual-Luciferase Reporter Assay System (Promega) following the manufacturer's instructions. The relative luciferase activity was calculated by the ratio of firefly and *Renilla* expression in each sample. All transfections were carried out in at least two independent experiments in triplicate. Data are expressed as mean values with standard errors, and were analysed by unpaired two-tailed t-test.

3.4.5 RT-qPCR

Reverse transcription was performed using 1 μg of total RNA, random primers and 20 U of reverse transcriptase (Promega) in a total volume of 50 μL. *ANKRD11*, *DNMT1* and *DNMT3B* expression were determined by RT-qPCR using IQ™ SYBR green supermix (Biorad) and primers listed in Table 3.2. Cycling condition were: 10 min at 95°C followed by 40 repeats of the following cycle: 95°C for 10 s, annealing at

the appropriate temperature for 10 s and extension at 72°C for 10 s. *β-actin* expression was used for normalisation of target gene expression.

3.4.6 SEQUENOM MassARRAY EpiTYPER for methylation analysis

DNA samples were bisulphite-treated and purified using the Epitect Kit (Qiagen) according to the manufacturer's protocol. Unconverted genomic DNA was included as a negative control. Primer design ensured the PCR amplification of completely converted DNA. Three overlapping regions (PF1, PF2 and PF3) of the bisulphite-treated *ANKRD11* promoter sequence were amplified using the PyroMark Kit (Qiagen) and primers in Table 3.2. As described previously, *in vitro* transcription and uracil-specific cleavage of the amplified products were undertaken before analysis of the samples by MALDI-TOF-MS (Wong et al., 2008).

Table 3.1 Cloning design for luciferase reporter system (pGL3-basic vector)

Name of the construct	*Template*	Forward primer (5'—3')	Reverse primer (5'—3')	Product size (bp)	Location
P1	*Hut78 genomic DNA*	AGCTGGACT GCGCTGACC GCACCTGTG	CACACACACACACACA CACTCGAGAAGCTTAG GGCTGCGCGGCTCCG CGACGGCTCAG	2306 (XhoI/ HindIII)	-2000 to +306 upstream sequence and exon 1
P2	*P1*	-	-	1339 (SacII/HindIII deletion of –661 to +306)	-2000 to -661 upstream sequence
P3	*P1*	-	-	877 (KpnI deletion of -2000 to -571)	-571 to +306 upstream sequence and exon 1
P4	*P1*	CACACACAC AACGCGTAG AACACAGGT GCACTTAGA ACAGGA	CACACACACAAGATCT GCTCCCGGTGCGGACG CTACTGAT	969 (MluI/BgLII)	-862 to +107 upstream and exon 1
P5	*P1*	CACACACAC AACGCGTAG GCCCAGGG	CACACACACACACACA CACTCGAGAAGCTTAG GGCTGCGCGGCTCCG	995 (MluI/HindIII)	-689 to +306 upstream sequence and

		GCTCTGGA	CGACGGCTCAG		exon 1
P6	*P1*	CACACACAC AACGCGTAG GCCCAGGG GCTCTGGA	CACACACACAAGATCT GAAGAGCTCTGGGCGG CTGCGAGGGGAGGGTA CCGGGGCAGCGGGGC GGTCGCTCGGGGCTCC CGCGC	146 (MluI/BglII)	-689 to -543 upstream sequence and methylation-sensitive
P6-CpG triple mutant	*P6*	CACACACAC AACGCGTAG GCCCAGGG GCTCTGGA	CACACACACAAGATCT GAAGAGCTCTGGGCGG CTGCGAGGGGAGGGTA CTTGGGCAGTTGGGTT GTCGCTCGGGGCTCCC GCGC	146 (MluI/BglII)	CpG triple mutant in the methylation-sensitive region
P6-double mutant	*P6*	CACACACAC AACGCGTAG GCCCAGGG GCTCTGGA	CACACACACAAGATCT GAAGAGCTCTGGGCGG CTGCGAGGGGAGGGTA CCGGGGCAGCTTTTTT TTTGCTCGGGGCTCCC GCGC	146 (MluI/ BglII)	First two CpGs mutant in the methylation-sensitive region
P6-CpG 3rd mutant	*P6*	CACACACAC AACGCGTAG GCCCAGGG GCTCTGGA	CACACACACAAGATCT GAAGAGCTCTGGGCGG CTGCGAGGGGAGGGTA CTTGGGCAGCGGGGC GGTCGCTC	146 (MluI/BglII)	CpG 3rd mutant in the methylation-sensitive region

Table 3.2 PCR primers used in this study

PCR	Target gene	Forward primer (5'—3')	Reverse primer (5'—3')	Product size (bp)	Location	T_{m}, °C
RT-qPCR	*ANKRD11* variant a; variant b [NM_013275.4]	AGCCAGGGTGACGAGAACAAGTC	CACACACAGGATCCTCAGTCGTCGTTGACGTCGACCATG	265	Exon 13	57
	ANKRD11 variant a	TGCAGCCTGCCAGGACTCTT	CGTCCTGCTCCTCACCCGAT	211	Exon 2 & 3	63
	DNMT1 variant 1 [NM_001130823.1]; variant 2 [NM_001379.2]	GCTGTGCCCGTCTGGCTGAG	TTCCGTGGGCGTTTCACGGG	199	Exon 27 & 28	57
	DNMT3B [NM_006892.3]	CCAACAACACGCAACCAGTGGT	TCCCCTCGGTCTTTGCCGTTGT	187	Exon 7 & 8	58
	β-actin [NM_001101.3]	TACCTTCAACTCCATCATGAAGTG	CCGGACTCGTCATACTCCTGCTTG	267	Exon 2	57
Sequenom methylation analysis	*ANKRD11* variant b [NM_013275.4]	aggaagagagGYGTGTTGGAGAATTAATTAGTAAGGT	cagtaatacgactcactatagggagaaggctCRAACCTAACRATTAATCTTTCCAAATA	488	-770 to -323 upstream sequence **(PF1)**	56

	ANKRD11 variant b [NM_013275.4]	aggaagagagGCGACGGAGGTAGTTAATTTAGTTGT	cagtaatacgactcactatagggagaaggctACRACCTCTAACTCCAAAACCCAA	349	-299 to +15 upstream sequence and exon 1 **(PF2)**	56
	ANKRD11 variant b [NM_013275.4]	aggaagagagTAGTYGTYGTTGGGTTTTGGAGTTAGAGGT	cagtaatacgactcactatagggagaaggctAAAAAAACCTACAACCRCAAAACC	464	-23 to +399 upstream sequence and exon 1 **(PF3)**	58

*lowercase denotes sequence tags added to facilitate downstream EpiTYPER analysis

T_m: annealing temperature

ANKRD11: Homo sapiens ankyrin repeat domain 11

DNMT1: Homo sapiens DNA (cytosine-5-)-methyltransferase 1

DNMT3B: Homo sapiens DNA (cytosine-5-)-methyltransferase 3 beta

β-actin: Homo sapiens actin, beta (ACTB)

3.5. Results

3.5.1 *ANKRD11* is downregulated in human breast tissues

We assayed the levels of *ANKRD11* mRNA in human invasive breast tumour tissues and normal samples using RT-qPCR (Fig. 3.1A). All but one of the tumour samples (T14) had a lower level of mRNA (3.9 ± 0.8, n=30) than the normal breast tissues (15.5 ± 7.5, n=5) ($p < 0.01$, Fig. 3.1A and B). Interestingly, the tumour sample with higher levels of *ANKRD11* expression was a papillary breast cancer (T14, Table 3.3). Notably, the primers used to assay levels of *ANKRD11* mRNA would bind both variant A [XR_123180.1] and B [NM_013275.4] that share common mRNA sequences at the 3′ end. In fact, variant A and B are translated to the same protein (2633 amino acids). However, the 5′UTR of variant A [XR_123180.1] (291 bp) is shorter than variant B (461 bp) and their 2 kb upstream sequences are slightly different. To determine if one of these variants was responsible for higher *ANKRD11* expression in T14, variant A-specific primers were designed. The result showed that the variant B [NM_013275.4] of *ANKRD11* was responsible for the higher expression in the single case of papillary carcinoma, T14 (Fig. 3.2).

Table 3.3 Clinical data of human breast samples in this study

Samples	ER	PR	HER2	MIB-1 count	Tumour type	Grade
T1	+	+	-	<20%	IDC/ ILC	1
T2	-	-	-	>30%	IDC	2
T3	+	+	++	>30%	IDC	3
T4	-	-	-	>30%	IDC	2
T5	-	-	-	>30%	IDC	3
T6	+	-	-	>30%	ILC	2
T7	+	+	+++	>30%	IDC	3
T8	+	+	-	>30%	IDC	2
T9	-	-	-	>30%	IDC	3
T10	-	-	+++	>30%	IDC	3
T11	+	+	+++	>30%	IDC	3
T12	+	+	++	20-30%	IDC	2
T13	+	-	-	<20%	IDC	1
T14	+	+	-	20-30%	IPC	1
T15	+	+	+++	<20%	IDC	2
T16	+	-	-	<20%	ILC	2
T17	+	+	+	<20%	IDC	1
T18	+	+	-	<20%	IDC	1
T19	+	+	+	<20%	IDC	3
T20	+	+	-	>30%	IDC	2
T21	+	+	-	<20%	ILC	1
T22	+	+	+++	>30%	IDC	3
T23	-	+	-	<20%	ILC	3
T24	+	+	+	<20%	IDC	1
T25	+	+	++	20-30%	IDC	2
T26	+	+	+	<20%	IDC	2/3

T27	+	+	-	>30%	ILC	2
T28	-	-	++	>30%	IDC	3
T29	-	-	+++	>30%	IDC	3
T30	+	+	+++	>30%	IDC	3

ER, PR, HER2, MIB-1 count, tumour type and grades of each tumour sample are shown.

Figure 3.1

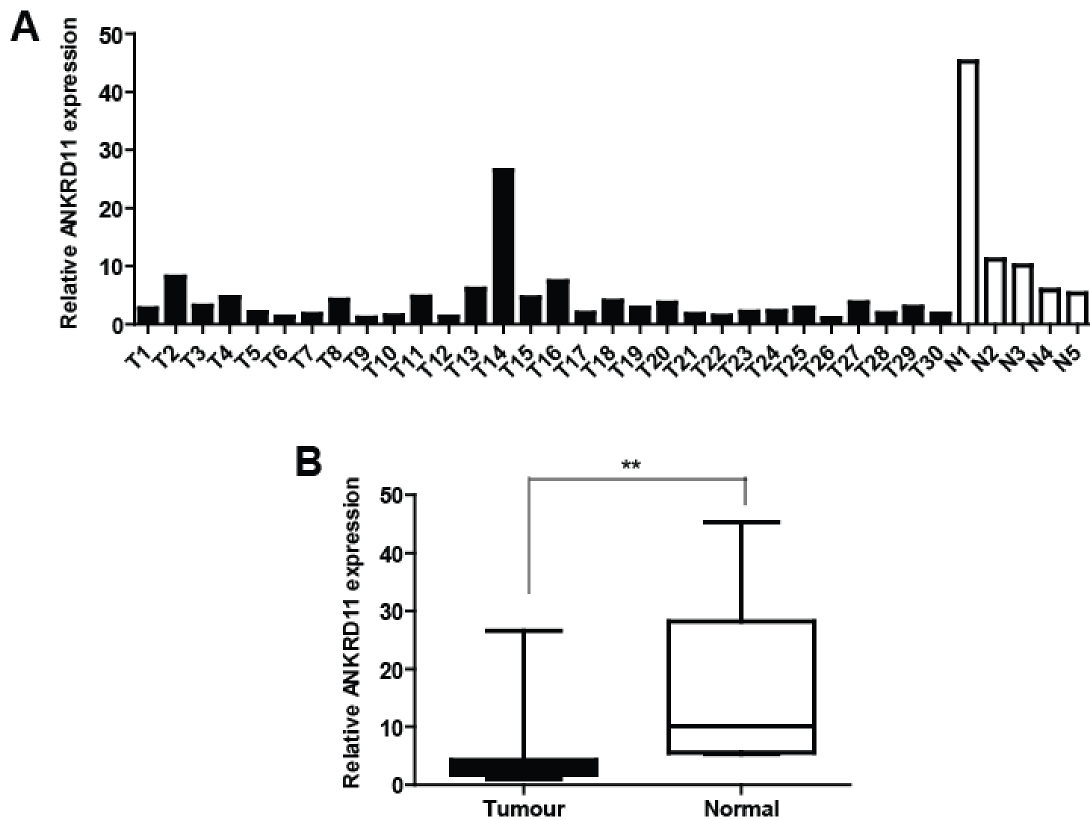


Figure 3.1 *ANKRD11* is downregulated in breast tumours. (A) The *ANKRD11* mRNA expression level in tumour (T) and normal (N) samples normalised to housekeeping gene *β-actin*. (B) The average mRNA levels of all tumour (n = 30) and normal samples (n = 5). An unpaired two-tailed t-test was used. The data are presented as mean ± SEM; ** = p < 0.005.

Figure 3.2

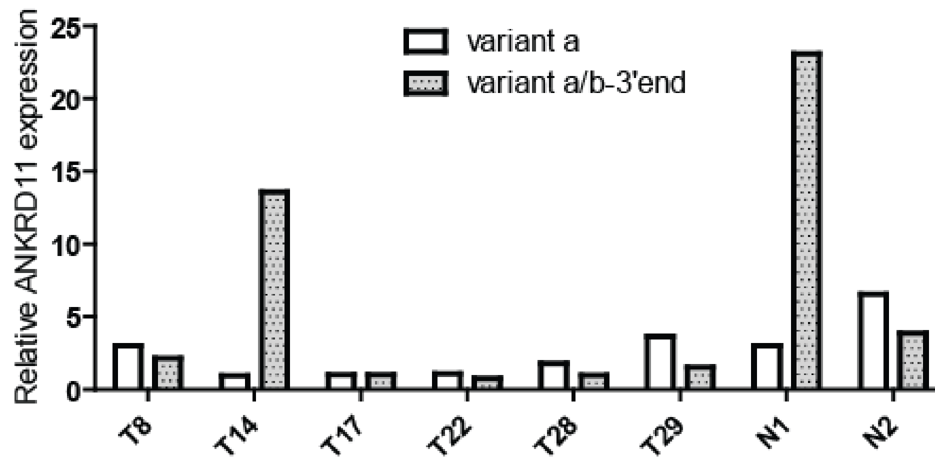


Figure 3.2 Variant B [NM_013275.4] is responsible for higher levels of ANKRD11 expression in T14 tumour and in N1 normal breast tissue. Primers specifically targeting variant A showed low expression of variant A in breast tissues samples. The *ANKRD11* expression level of each sample was normalised to *β-actin*.

3.5.2 ANKRD11 expression in breast cell lines is restored by DNMT inhibitors

We next measured levels of *ANKRD11* mRNA in breast cancer cell lines. MDA-MB-231 (ER-), MCF-7 (ER+), MDA-MB-468 (ER-), ZR75-1 (ER+) and the immortalized breast cell line MCF10A (ER-) have low levels of *ANKRD11* expression when compared with normal breast tissue (N1, N2; Fig. 3.3A).

To investigate whether *ANKRD11* inactivation in breast cell lines is caused by epigenetic modification such as DNA methylation, we treated these cell lines with demethylating agents (DNMT inhibitors) (Lim et al., 2011). The breast cell lines showed variable response to two DNMT inhibitors, decitabine and zebularine. Modest increases in levels of *ANKRD11* expression were observed in decitabine treated MCF-10A and MDA-MB-468 (Fig. 3.3B and C). MDA-MB-231, MCF-7 and ZR75-1 showed no response to decitabine treatments (Fig. 3.3D-F). Zebularine was the most effective agent as it induced at least 5-fold increases in *ANKRD11* expression at the highest concentration used (500 μM), in all cell lines except MCF-7 (Fig. 3.3B-F). These findings suggest zebularine and decitabine responses are cell-type dependent. Taken together, these results suggest that *ANKRD11* could be downregulated by DNA methylation as *ANKRD11* gene activity was induced in response to DNMT inhibitors.

Figure 3.3

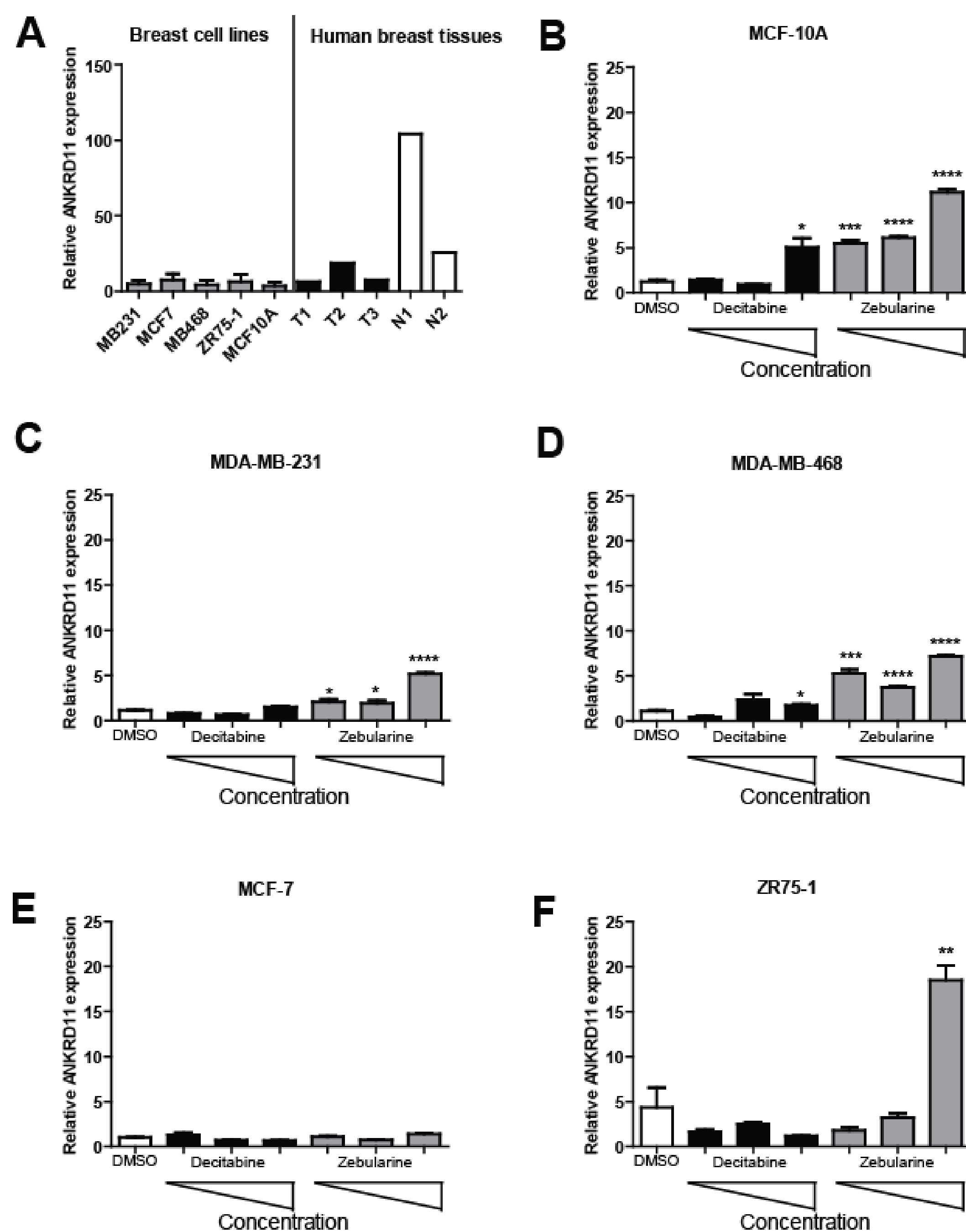


Figure 3.3 *ANKRD11* is upregulated in breast cell lines after treatment with DNMT inhibitors. (A) The relative expression of *ANKRD11* was measured in breast cell lines and human breast tissues as described in Fig. 3.1A. *ANKRD11* mRNA expression is shown relative to vehicle control in (B) the immortalised non-malignant breast cell line, MCF-10A and, breast cancer cell lines: (C) MDA-MB-468, (D) MDA-MB-231,

(E) MCF-7 and (F) ZR75-1. The cells were treated with 1, 10, 50 μM decitabine or 50, 100, 500 μM zebularine for 72 hours. The relative *ANKRD11* expression was normalised to *β-actin*. Unpaired two-tailed t-test for all statistical analysis compared to vehicle control. * = $p < 0.05$, ** = $p < 0.005$, *** = $p < 0.001$.

3.5.3 Identification of *ANKRD11* promoter

Since demethylating agents can activate the expression of *ANKRD11* in breast cell lines, promoter activity of this gene was characterized in order to further study its methylation status. The *ANKRD11* gene contains 13 exons and is transcribed from within exon 3 (Fig. 3.4A). A CpG island was identified that extends from exon 1 to 800 bp upstream of the transcription start site. A 2 kb region identified as a putative promoter region was tested for its transcriptional activity using a dual-luciferase reporter assay (Fig. 3.4B). A 2317 bp fragment of *ANKRD11*, including exon 1 and 2 kb upstream (designated as P1), was cloned into a luciferase reporter vector, together with various sub-fragments of P1 (Fig. 3.4B). The constructs P4 (-862 to +107) and P5 (-689 to +306) showed the highest promoter activity. However, constructs P1 and P3 exhibited modest levels of transcriptional activity that were completely abolished in P2, which lacked the -660 to +308 bp region. These results suggest that sequences regulating the promoter activity of *ANKRD11* are located within the -689 to +107 bp region (Fig. 3.4B).

Figure 3.4

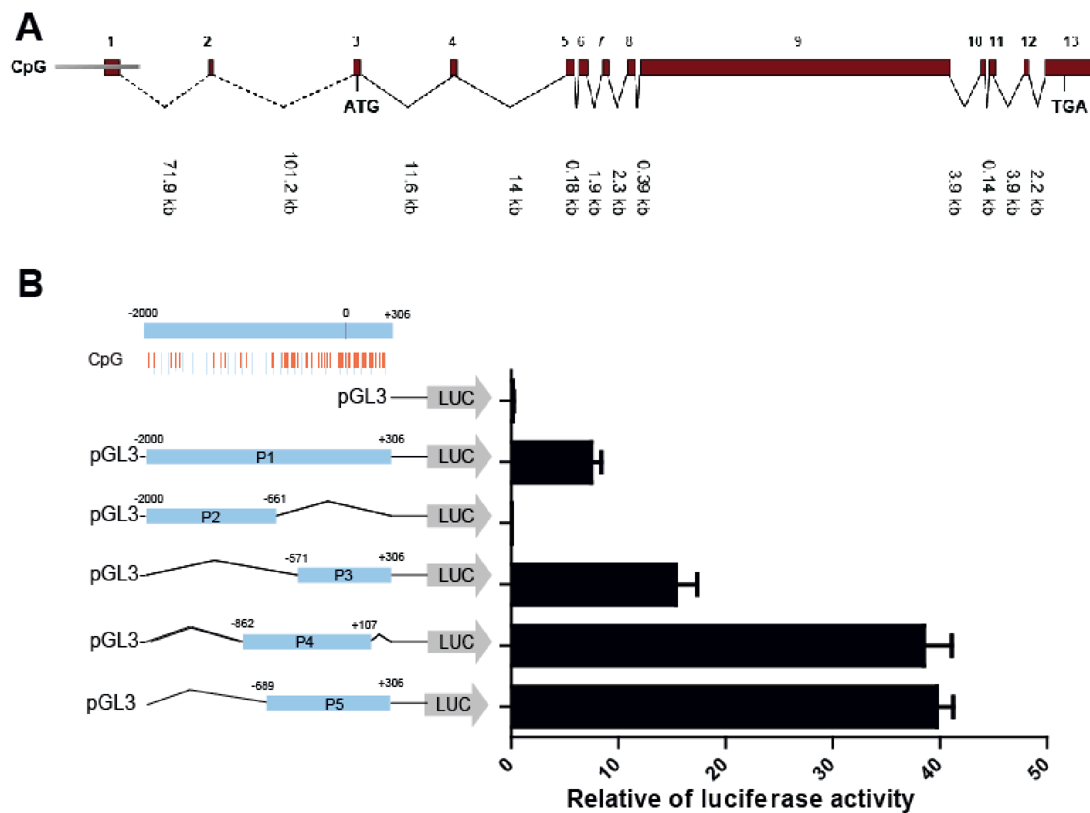


Figure 3.4 Characterisation of the *ANKRD11* promoter. (A) Genomic organisation of *ANKRD11*. The 223 kb *ANKRD11* gene is transcribed to a 9.3 kb mRNA. (B) The transcriptional activity of the upstream region (5′ flanking region and exon 1) of *ANKRD11* was measured. CpG dinucleotides are shown as vertical bars. Promoter constructs P2, P3, P4 and P5 represent different parts of P1. Values represent averages of triplicate treatments from at least two independent experiments. ** = $p < 0.005$.

3.5.4 The *ANKRD11* promoter is methylated in breast cancer

Following identification of a putative *ANKRD11* promoter region, the total methylation status of three overlapping regions of the promoter (PF1 to PF3) was analysed in breast tumour, and normal breast samples using MALDI-TOF-MS (Fig. 3.5A). SEQUENOM MassARRAY EpiTYPER employs base-specific cleavages followed by MALDI-TOF-MS to quantify DNA methylation of CpG sites within a target region. DNA methylation of CpG sites across each amplicon, PF1 (-770 to -323 bp), PF2 (-299 to +15 bp) and PF3 (-23 to +399 bp), were averaged to identify the amplicon with higher methylation in breast tumour samples. A significantly higher methylation was observed in the PF1 region compared with regions PF2 and PF3 (Fig. 3.5B). This suggests that the promoter region between -770 to -323 bp contains methylated CpGs that may be responsible for the aberrant *ANKRD11* expression in breast cancer.

3.5.5 Site-specific methylation of *ANKRD11* promoter

Since the PF1 region was the significantly methylated part of the *ANKRD11* promoter, 17 fragments (units) of PF1 were analysed by mass spectrometry (Fig. 3.5C). A comparison of DNA methylation status in seven normal and 25 breast cancer samples revealed that differences in methylation between normal and tumour breast tissues were localised to CpG unit 7. CpG unit 7 of PF1 is a 19 bp fragment that includes three CpG sites (-582 to -574 bp). This unit was highly methylated in 36% (9/25) of the tumour samples analysed (Fig. 3.5D).

We also investigated if there was any relationship between *ANKRD11* methylation status and clinical markers in breast cancer samples. The clinical data showing ER, PR, HER2 and MIB-1 status of the breast tumours is given in Table 3.3. Methylation at CpG unit 7 was observed to be significantly associated with HER2 negativity ($p<0.05$) (Fig. 3.5E), but was not associated with tumour grade, ER, PR or MIB-1 status (Fig. 3.6). In human tumour samples, there was a trend for high levels of methylation at CpG unit 7 to be associated with lower levels of *ANKRD11* expression (Fig. 3.5F). However, some tumour samples displayed low *ANKRD11* expression in the absence of elevated methylation, suggesting that in these samples *ANKRD11* is downregulated by alternative mechanism.

A similar trend was also seen in the breast cell lines. For example, the three cell lines with the higher level of methylation at CpG unit 7, MDA-MB-468, MDA-MB-231 and MCF10A, have a relatively low level of *ANKRD11* expression (Fig. 3.5G). These cell lines showed a reduction in methylation at CpG unit 7 after treatment with 500 μM of zebularine (Fig. 3.5H), which was previously shown in Fig.

3.3 as the most effective dose to increase *ANKRD11* expression. No reduction was observed in ZR75-1 and MCF-7 cells due to the low initial methylation level at this locus (Fig. 3.5H). Consistent result was found in MCF-7 cells where expression of *ANKRD11* was not increased by treatment with zebularine (Fig. 3.3E). In contrast, there was an elevated expression of *ANKRD11* in ZR75-1 cells after treatment with 500 μM of zebularine (Fig. 3.3F). Although there was a growth reduction, these zebularine-treated ZR75-1 cells were relatively viable when examined by light microscope (*data not shown*). A lack of association of the methylation level of *ANKRD11* with its gene expression in the ZR75-1 cells suggests the involvement of an alternative mechanism of downregulation such as chromatin remodelling. This is because epigenetic drugs such as zebularine can also re-express genes, which were previously silenced by histone modifications (Halaban et al., 2009, Scott et al., 2007, Radhakrishnan et al., 2008, Savickiene et al., 2012, Lavelle et al., 2006). Overall, these data suggested a relationship between specific methylation at CpG unit 7 of the *ANKRD11* promoter and total *ANKRD11* expression.

Figure 3.5

Figure 3.5 Methylation patterns of the *ANKRD11* promoter in human breast cancer. (A) Location and (B) methylation analysis of three regions (PF1, PF2 and PF3) within the *ANKRD11* promoter, in breast tumours. Each bar represents the average methylation level of all CpG units in each amplicon (PF1 = 17 units, PF2 = 15 units, PF3 =10 units). The statistical distribution of PF1 versus PF2 is $t = 3.6$, $df = 143$; PF1 versus PF3 is $t = 2.4$, $df = 119$; and PF2 versus PF3 is $t = 0.9$, $df = 120$. (C) Site-specific methylation levels of *ANKRD11* promoter region PF1. The % methylation of tumours (n=25) and normal samples (n=7) was averaged in each CpG units. The CpG units are as defined in Fig. 3.7. The methylation pattern of PF2 and PF3 are showed in Fig. 3.8. The statistical distribution of the normal and tumour samples in CpG unit 7 is $t = 2.4$, $df = 30$. (D) Specific methylation levels at CpG unit 7 of PF1 in tumour and normal samples. Tumours (black), normal (white) and blood samples (grey). T5, T18, T21, T23, T30, N3, N4 and N5 are not shown. (E) The relationship of human epidermal growth factor receptor (HER2) status and methylation level at unit 7 ($t = 2.2$, $df = 23$). (F) Promoter methylation of *ANKRD11* promoter at CpG unit 7 and *ANKRD11* mRNA expression in human breast tissues ($t = 1.5$, $df = 23$). The data from T14 (a different type of breast tumour) and N2 were treated as outliers. High= samples with >50% methylation at unit 7; low = <50% methylation at unit 7. There was no significant relationship between methylation and expression. (G) Correlation of promoter methylation of *ANKRD11* at unit 7 and relative *ANKRD11* expression and (H) PF1 epigram of breast cell lines treated with 500 μM of zebularine (Zeb) for 72 hours. Bracket: three CpGs in unit 7. An unpaired two-tailed t-test was used for all statistical analysis. * = $p < 0.05$. *** = $p < 0.001$.

Figure 3.6

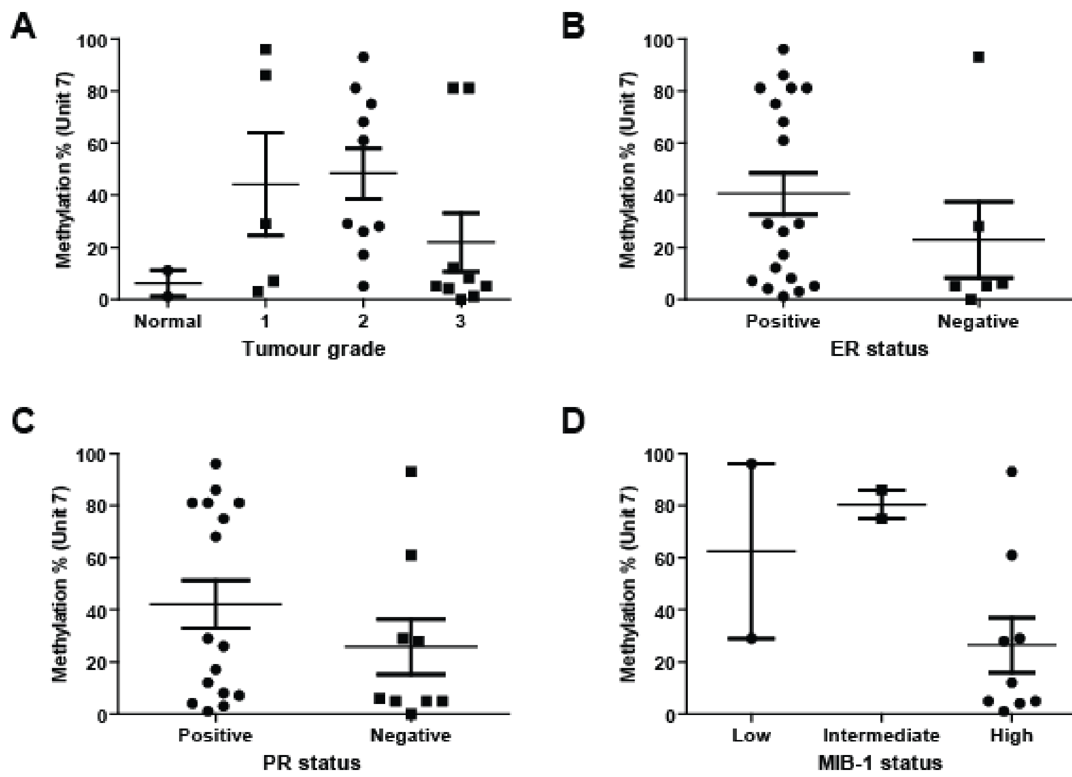


Figure 3.6 Relationship of (A) tumour grade, (B) ER, (C) PR and (D) MIB-1 status and methylation level at unit 7. An unpaired two-tailed t-test was used for all statistical analysis. These were statistically insignificance. The data are presented as mean ± SEM.

Figure 3.7

PF1

-770

GCGTGTTGGAGAATTAATTAGTAAGGTAGGGAATGCGGTTCGTGGGCGTTTGGTTT

Unit 1

TTTTA|AGCGGTTT|AGGTTTTAGAGCGCGAGGTTTAGGGGTTTGG|AG

Unit 2 Unit 3 Unit 4

TCGCGGTCGCG|ACGCGTTCG|AGGGTCGTTTTTTCG|ACGGTAGAGTA

Unit 5 Unit 6 Unit 7

AGAGTT|ATCGCGCGGG|AGTTTCG|AGCG|ATCGTTTCGTTGTTTCGGT|A

Unit 8 Unit 9 Unit 10

TTTTTTTTTCGT|AGTCGTTTAGAGTTTTTT|ATGTTTCGTG|ACGTTCGTT|

Unit 11 Unit 12 Unit 13

AGGGTAGCGCGGTCGG|AGGTCGGGTCGG|ACGTGGTTCG|AG|ATGTTCG

Unit 14

GGGGT|AGGTAGAAGATTACGGGTACGCGATAGGTA|ATCGTCGTTTCG

GTTTT|AGAGGTGGACGTTTTGTTTTTTTGCGTTTTAATATTAGATTTTGTT

Unit 15 Unit 16 Unit 17

TTTAGACG|ATATTTTTGTTGTTTTCGGT|ATTTGGAAAGATTA|ATCGTT|AGGTTCG

-323

PF2

-299

Unit 1 Unit 2

GCGACGGAGGTAGTTAATTT|AGTTGTTCGTT|ATTTTTAGTGTCGT|ATGCGTTTTT|

Unit 3 Unit 4 Unit 5

ACGTAGGATTTTGTTTAATCGTT|ATCGGG|ACGTTTTTTT|AGTTTCGTTT

Unit 6

TCGG|ATCGTTAGAGAGGTTGTTA|ATTTCGTTTTTTTTTTT|ATTTTATT|

Unit 7 Unit 8 Unit 9

AGCGT|ATTAATTT|ACGGCGTTGTG|ACGTGT|ATAGTTTTTGCGGTCGGT

Unit 10 Unit 11 Unit 12

CGGGGCGGGTTA|ATGGCGGTGGCGG|AT|ACGGCGGGTT|A|ATGGGCGG

Unit 13 Unit 14 Unit 15

G|AGCGTTTT|ATGCGGTCGGTTTTCGTCGT|AGTCGTCGTTGGGTTTTGG|AGT

TAGAGGTCGTTTTGAG

+15

PF3

-23

Unit 1 Unit 2

T|AGTCGTCGTTGGGTTTTGG|AGTTAGAGGTCGTTTTGAG|ACGGTGCGCG|ATGGA

Unit 3 Unit 4

TCGAGGGTTTT|AGTCGGGG|AGGCGTCGTCGTCG|AGTTCGCGGTTAG|A

Unit 5 Unit 6

CGTTTT|ATTAGTAGCGTTCGTATCGGG|AGTCGCGGTTTTCGTTCG|AGT

CGTGGGCGCGTTCGAGGGGCGGGTTCGTTTTTCGTCGTTTTTCGT|AGT

Unit 7

TTTGTCGGGTTCG|AGTTCGCGTCGTCGTCGTCGTCGTTTTGTCGTTCG

GGTCGCGCGGTTCGGGAAACGCGGTCGCGGGTTGTATGGGT|AGCGTT

Unit 8 Unit 9

CGCGTTTCGTCGTTG|AGTCGTCGCGG|AGTCGCGTAGTTTTCGGAGTA

Unit 10

CGGTGAGAGGCGTCGTTGGTTTGGGGGCGGTGGTCGGGGCGGGT|ACGG

GGT|ATTCGCGCGGTTTTGCGGTTTGTAGGTTTTTTT

+399

Figure 3.7 Amplicon design and the target region for methylation analysis. Bisulfite treated sequence of *ANKRD11* promoter regions: PF1; PF2; PF3. [T bold: cytosine from non-CG converted to T; *italic smaller font*: primer target sequence; all CGs: bold; CG underlined: analysed CGs; |Unit|: fragment with different mass and size generated by enzymatic base specific cleavage.]

Figure 3.8

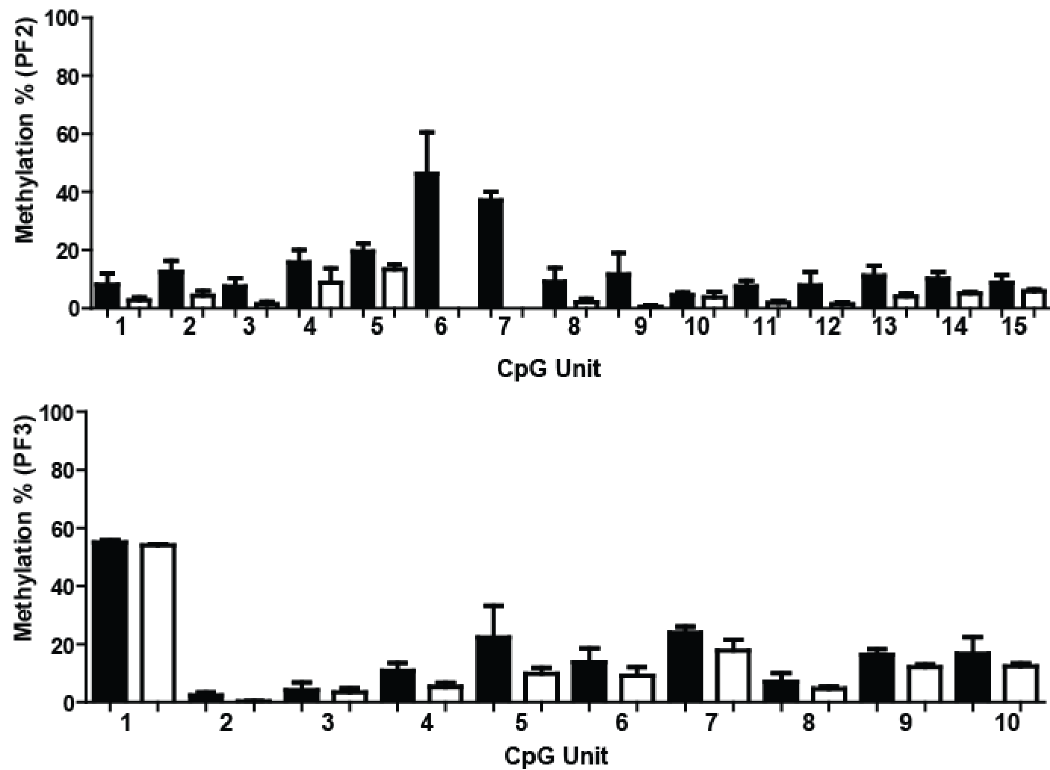


Figure 3.8 Average methylation levels of all CpG units in (A) PF2, T, n = 6; N, n = 4; and (B) PF3 T, n = 5; N, n = 4. Tumour (T, black) and normal (N, white). The % methylation of tumours and normal samples was averaged in each CpG units. An unpaired two-tailed t-test was used for all statistical analysis. No significant difference was found between normal and tumour samples in all units. The data are presented as mean ± SEM.

3.5.6 Site-specific mutation and DNA methylation silence *ANKRD11* expression

To examine whether the activity of the three CpGs in the unit 7 methylation-sensitive region (-582 to -574 bp) regulate *ANKRD11* expression, specific CpGs in the P6 construct (-689 to -543 bp) were either methylated by specific enzymes (Fig. 3.9A) or mutated by site-specific mutagenesis (Fig. 3.9B). In *in vitro* methylation study, methylase enzymes were used to methylate specific CpG sites in the P6 promoter fragment. Promoter activities of each fragment were then assessed. The transcriptional activities of all *in vitro* P6 constructs were significantly reduced after being methylated (Fig. 3.9A). Methylation of the third CpG in unit 7 by *Hpa*II methylase more strongly repressed the promoter activity compared to the *Hha*I methylated P6, which methylated the CpG adjacent to unit 7.

Site-directed mutagenesis of the first two CpGs and all three CpGs in unit 7 significantly reduced transcriptional activity (Fig. 3.9B). However, mutation of the third CpG alone did not show any relationship in transcriptional regulation of *ANKRD11*, suggesting the critical region was 10 bp region consisting the first two CpGs.

Figure 3.9

A aggcccaggggctctggagccgcggccgcgacgcgcccgaggg

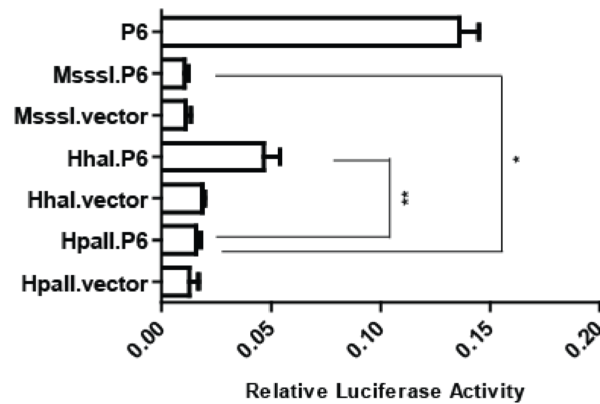
ccgttctcttcgacggcagagcaagagtcaccgcgcgggagcc

ccgagcgACCGCCCCGCTGCCCCGGTaccctcccctcgcagcc

gcccagagctcttc

CG: M.SssI

(HhaI sites: **gcgc**; HpaII site: **CCGG**; positions −582 and −574 marked)



B Site-mutagenesis:

P6 (wild-type)	gACCGCCCCGCTGCCCCGGT
CpG triple mutant	gAC**AA**CCC**AA**CTGCCC**AA**GT
CpG double mutant	**AAAAAAAAA**GCTGCCCCGGT
CpG 3rd mutant	gACCGCCCCGCTGCCC**AA**GT

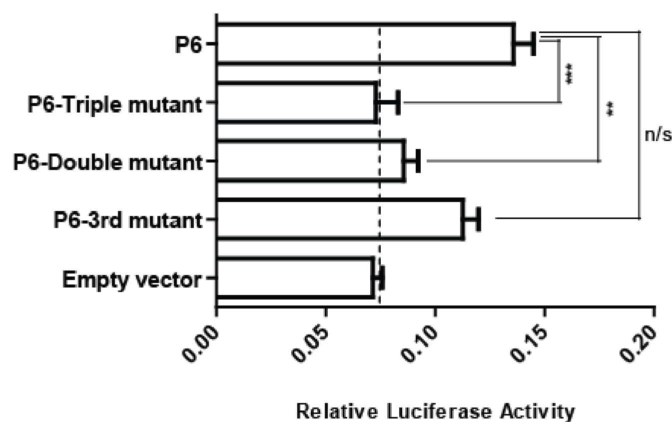


Figure 3.9 In vitro methylation and site-directed mutagenesis of *ANKRD11* promoter.

(A) *In vitro* methylation target sequence and the luciferase activity of *ANKRD11*

promoter (pGL3-P6) located from -689 to -543 bp upstream of the transcription start site. (B) Site-directed mutagenesis of specific sequences and luciferase activity of pGL3-P6. Unpaired two-tailed *t*-tests were conducted on each construct compared to the original P6. Recognition sequences for *HhaI* (5′-GCGC-3′) and *HpaII* (5′-CCGG-3′) are shown in bold. Recognition sequences for *M.SssI* (5′-CG-3′) are underlined. Unit 7 is shown in uppercase text. * = $p < 0.05$, ** = $p < 0.005$, *** = $p < 0.001$, n/s = not significant.

3.5.7 Relationship of DNMTs and *ANKRD11* expression

DNMT1 and *DNMT3B* are responsible for the maintenance and establishment of DNA methylation patterns, respectively (Chen et al., 2003). *DNMT3B* is frequently over-expressed in breast tumours (Bestor, 2000, Girault et al., 2003). To identify whether *DNMT1* and *DNMT3B* have roles in regulating *ANKRD11* expression, mRNA levels of *DNMT1* and *DNMT3B* were measured in the breast tumour and normal samples. In our dataset, *DNMT3B* was over-expressed in 30% of the tumour samples and was very high in higher grade tumours (Fig. 3.10) (Girault et al., 2003). However, there was no relationship between the *DNMT3B* and *ANKRD11* expression (Fig. 3.10). In addition, *DNMT1* expression varied among breast tumours, normal breast tissues and normal blood samples (Fig. 3.10) consistent with the published data (Girault et al., 2003).

Figure 3.10

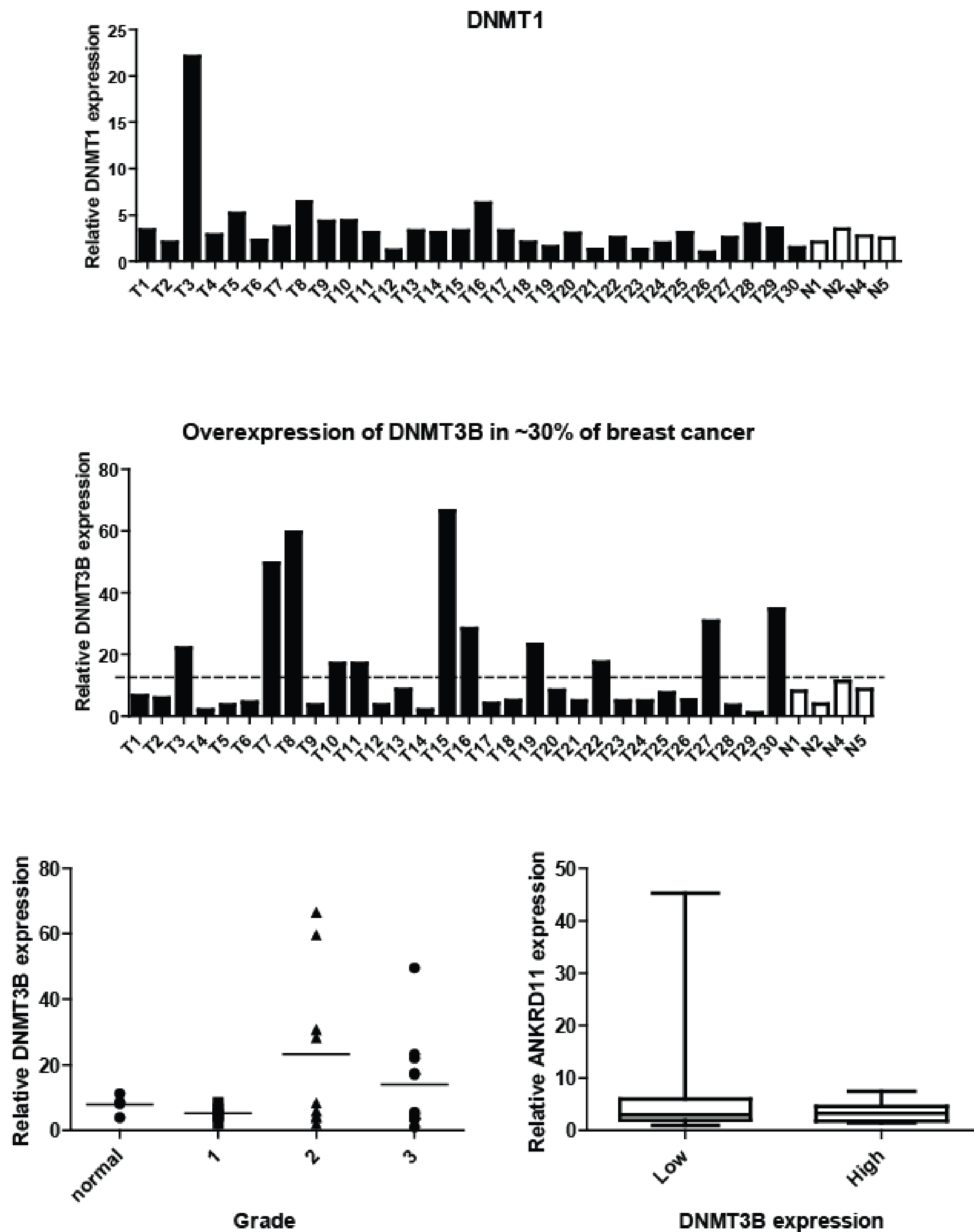


Figure 3.10 Levels of *DNMT1* and *DNMT3B* mRNA were assayed in human breast tissues. N = normal breast sample from reduction; T = breast tumour. N3 is not shown due to insufficient sample. The relationships of tumour grades, *ANKRD11* and *DNMT3B* expression are also shown. An unpaired two-tailed t-test was used for all statistical analysis. No significant difference was found between expression of

DNMT3B and different tumour grades, or expression of *DNMT3B* and *ANKRD11*. The data are presented as mean ± SEM.

3.6 Discussion

In this study, mRNA expression of *ANKRD11* was shown to be uniformly downregulated in 23 cases of infiltrating ductal, five cases of lobular and a case of mixed ductal and lobular breast cancer tissues compared to normal breast tissues. The exception was a single case of invasive intracystic papillary cancer, a breast cancer subtype that has a good prognosis (Grabowski et al., 2008). Additional studies are needed to further investigate the possible relationship of *ANKRD11* expression and papillary breast cancer.

The use of DNMT inhibitors such as decitabine and zebularine can inhibit DNMTs and subsequently activate methylation-silenced genes (Yoo and Jones, 2006). The *ANKRD11* mRNA expression was shown to be upregulated in breast cell lines after treatment with different concentrations of these inhibitors, with zebularine showing the maximal response. This suggests DNA methylation is a likely mechanism for downregulation of *ANKRD11* expression.

Following identification of the *ANKRD11* promoter, we found a region located between -689 and +107 bp has enhanced *ANKRD11* transcription. Analysis of this region in breast tumour samples identified three CpGs within the region -582 to -574 bp that were hypermethylated, however, it was not significantly related to *ANKRD11* expression.

In the *in vitro* methylation study, the methylation at specific CpGs of the *ANKRD11* promoter was shown to reduce the transcriptional activity of *ANKRD11*, especially methylation at the third CpG. This supports a relationship of specific

methylation and transcriptional activity of *ANKRD11*. However, site-directed mutagenesis of the third CpG did not affect the transcriptional activity, suggesting methylation and mutation of this region could be independent mechanism in regulating the transcription of *ANKRD11*. The site-directed mutagenesis studies defined a region containing the two CpG dinucleotides as critical in the methylation-sensitive regulation of *ANKRD11* transcription. *In silico* analysis identified GLI1 as a possible transcription factor that binds to this sequence 5′-GACCgCCCcg-3′ (Ji et al., 2006), but additional functional analysis is required to confirm such a relationship.

The overexpression of *DNMTs* has been proposed as a mechanism for aberrant genome methylation (Girault et al., 2003). There was little variation in *DNMT1* levels among our breast tumour samples. However, approximate 30% of tumours showed high levels of *DNMT3B* expression. In the methylation analysis, there was no significant relationship between specific methylation and *ANKRD11* expression in human tumour samples, although there was a trend for low *ANKRD11* expression and specific methylation of *ANKRD11* in breast cancer cell lines. These findings speculate that mechanisms other than promoter methylation may be responsible for low *ANKRD11* expression in some breast tumours.

By comparing clinical data with specific methylation of *ANKRD11*, the pattern or methylation status of *ANKRD11* promoter region PF1 was shown to be associated with HER2 status. As silencing of tumour suppressor genes through promoter hypermethylation is known to be a frequent and early event in carcinogenesis (Brooks et al., 2009), methylation status of *ANKRD11* could be a candidate marker for early tumour detection.

In conclusion, this study is the first to identify *ANKRD11* promoter specific methylation in breast tumours. The frequently observed specific methylation of the *ANKRD11* promoter and downregulation of *ANKRD11* mRNA levels in breast tumours point toward an association between the inactivation of this tumour suppressor gene and aberrant breast growth.

3.7 Acknowledgements

This work has been supported by the Victorian Cancer Agency, the National Health & Medical Research Council, Australia, and the Victorian Government's Operational Infrastructure Support Program.

CHAPTER 4: DIFFERENTIAL EXPRESSION OF *ANKRD11* AND *GLI1* IN DUCTAL CARCINOMA *IN SITU* AND INVASIVE CARCINOMA OF THE BREAST

4.1 Foreword

In the previous chapter, the methylation of a small 19 bp region of the *ANKRD11* promoter between -582 to -574 bp was shown to be correlated with expression of *ANKRD11* in breast cancer cell lines. This correlation was not significant when a group of 25 breast tumours was analysed, although there was a trend for lower expression of *ANKRD11* when the 19 bp region was methylated. It is suggested that this lack of significant correlation could arise from the presence of normal stromal components and other cells in breast cancer biopsies. It is also possible that *ANKRD11* is downregulated in some tumours by other mechanisms than promoter methylation.

In HER2 negative breast cancer significant higher level of methylation of the *ANKRD11* promoter was observed at the 19 bp region. This region of the promoter sequence contained a putative GLI1-DNA binding site. In breast cancer, GLI1 was previously identified to be associated with increased invasion and migration by upregulating expression of MMP-11 (Matrix metalloproteinase 11), which has a role in breaking down components of the extracellular matrix (Kwon et al., 2011). It was speculated that GLI1 acts to transform cells by binding to its cognate DNA sequence

binding sites and modulating the transcription of a set of genes (Kinzler and Vogelstein, 1990). In this chapter, the relationship between *ANKRD11* and *GLI1* in breast cancer is investigated. The role of GLI1 is also assessed in the clinical samples previously used for *ANKRD11* analysis.

4.2 Abstract

ANKRD11 is a putative tumour suppressor and a p53-coactivator. *ANKRD11* has been shown to be upregulated by treatment with the demethylating agent zebularine in breast cancer cell lines. Although ~60% (15/25) of human breast tumour samples were methylated, there is no significant direct relationship between methylation of the *ANKRD11* promoter and level of mRNA expression in breast cancer samples, suggesting the involvement of additional alternative gene silencing mechanisms in some tumours (Chapter 3). *In silico* analysis of the restricted methylated region in the *ANKRD11* promoter revealed the presence of a putative GLI1 binding site.

In this study the relationship between the GLI1 transcription factor and *ANKRD11* transcriptional activity was investigated. Dual luciferase reporter assays did not support the functionality of the putative GLI1 binding site in the 19 bp region previously pin-pointed by methylation studies. Nevertheless, endogenous expression of *ANKRD11* showed a modest increase in MCF10A cells when GLI1 was overexpressed. In addition, a highly significant linear correlation was found between *ANKRD11* and *GLI1* mRNA expression in normal human and breast tumour tissues. This suggests that either functional GLI1 binding sites are present elsewhere on the *ANKRD11* promoter, a downstream target controlled by GLI1 controls the expression of *ANKRD11* or *GLI1* and *ANKRD11* are both controlled by a common unknown factor.

Interestingly, expression of both *ANKRD11* and *GLI1* were consistently highly elevated in ductal carcinoma in situ (DCIS) compared to normal breast and invasive

carcinoma of the breast (IBC). The levels of *GLI1* and *ANKRD11* expression in all 33 IBC samples were consistently repressed. We speculate high levels of *GLI1* and *ANKRD11* are associated with the early stages of breast cancer but elimination of expression is required for progression to invasive ductal carcinoma. Additional studies are required to confirm these proposals.

4.3 Introduction

In 1987 *GLI1* (glioma-associated oncogene family member 1) gene, which was previously known as *GLI*, was first identified as a highly amplified oncogene in glioblastomas (Kinzler et al., 1987). Later, GLI1 was found to be an important factor for proper embryonic development and differentiation (Ingham and McMahon, 2001). The GLI1 nuclear protein of 1106 amino acids consists of five highly conserved C_2H_2-Krüppel-like zinc finger domains and binds to DNA in a GACCACCCA-like motif (Kinzler et al., 1988). *GLI1* and two related genes *GLI2,* and *GLI3* (glioma-associated oncogene family members 2, 3) constitute a small family of proteins that share high conservation in their zinc finger domains. GLI2 (1586 amino acids) and GLI3 (1596 amino acids) are normally cytoplasmic proteins but can also bind specifically to genomic DNA at the GLI1-binding sites (Tanimura et al., 1993, Ruppert et al., 1990).

In humans GLI1 is a strong transcriptional activator, GLI2 has both activator and repressor functions, and GLI3 is mostly a repressor (Hynes et al., 1997, Sasaki et al., 1999, Karlstrom et al., 2003). While GLI1 contains only a C-terminal transcriptional activation domain, both GLI2 and GLI3 possess C-terminal activator and N-terminal repression domains (Dai et al., 1999, Sasaki et al., 1999). The major role of the GLI family of transcription factors is in the Hedgehog (Hh) signalling pathway, which is a major regulator of normal development (Hynes et al., 1997, Roelink et al., 1994, Chang et al., 1994, Echelard et al., 1993), and dysregulation of the pathway is a significant contributor to human diseases such as skin, colorectal and brain cancers (Dahmane et al., 1997, Buscher and Ruther, 1998, Bian et al., 2007). Therefore, the balance between GLI1, GLI2 and GLI3, which have different and yet

overlapping biochemical properties, determine regular development in the responding cells (Ruiz i Altaba et al., 2007, Eichberger et al., 2006).

The GLI1 is normally localised in the cytoplasm but it can be translocated to the nucleus where it functions by transactivating target genes. The nuclear translocalisation of GLI1 requires binding to one of the Hh ligands; sonic Hh, Indian Hh or Desert Hh, and to the transmembrane receptor Patched (PTCH). As a consequence, smoothened (SMO), which initiates the release of GLI1 from cytoplasmic proteins fused and Suppressor of fused (SuFu) for GLI1 nuclear translocation, is relieved from PTCH-induced inhibition (Kinzler and Vogelstein, 1990, Barnfield et al., 2005).

In a recent immunohistochemistry-based study higher levels of GLI1 were detected in DCIS and in IBC than in normal breast tissue (ten Haaf et al., 2009). DCIS of the breast is a pre-invasive type of breast cancer and a lesion composed of neoplastic ductal cells, without stromal invasion, in which the complete malignant phenotype such as uncontrolled proliferation, angiogenesis and metastasis have not been fully expressed. The definition of DCIS is based on phenotype rather than the genotype of the tissues (Silverstein, 1998).

Recently, GLI1 was found to promote invasiveness and migration by upregulating MMP-11, which is responsible for breaking down extracellular matrix and basement membrane in ER negative breast cancer (Kwon et al., 2011). Other important cancer-related targets of GLI1 include FOXM1 (Forkhead box protein M1) (Teh et al., 2002) and OPN (osteopontin) (Das et al., 2009). FOXM1 and OPN are

often overexpressed in multiple cancers and implicated in metastasis (Raychaudhuri and Park, 2011, Wai and Kuo, 2008). Collectively, these studies suggested that aberrant expression of GLI1 can promote cancer development by upregulating downstream targets. In addition, a high level of GLI1 protein expression is also associated with unfavourable overall survival in patients with invasive breast cancer (ten Haaf et al., 2009, Thomas et al., 2011). In animal models, transgenic expression of GLI1 in the mouse mammary gland is associated with the appearance of hyperplasia, impaired development of terminal end buds and results in mammary tumour development (Fiaschi et al., 2009). Taken together, the protein expression of GLI1 can be considered as an important indicator for determining the aggressiveness of breast cancer. However, the use of GLI1 protein as a biomarker is limited by the time-consuming immunohistochemistry scoring that is required (Hicks, 2011).

So far, only a few studies have looked at the mRNA expression of *GLI1* in human breast tissues. One study involved comparing the expression of *GLI1* in five matched pairs of tumour tissues, however, there was no significant relationship between tumour and matched normal due to the low sample numbers (ten Haaf et al., 2009). A further study specifically quantified *GLI1* message in the isolated specimens that contained positive expression of GLI1 protein and showed *GLI1* mRNA expression in DCIS ($n = 9$) was slightly lower than IBC ($n = 16$) ($p < 0.05$) (Souzaki et al., 2011). Collectively, there is a lack of extensive reported data of *GLI1* mRNA levels in human breast cancer.

We have recently reported that the expression levels of the tumour suppressor cell lines are correlated with the presence of specific-site methylation of the

ANKRD11 promoter (Chapter 3). ANKRD11 is generally downregulated in breast cancers compared with normal breast tissues.(Chapter 3) (Lim et al., 2012). In general tumours with specific-site methylation of the *ANKRD11* promoter have a trend towards lower average values of *ANKRD11* expression. It is considered that the lack of a statistically significant correlation between expression of *ANKRD11* and its specific-site promoter methylation is related to the presence of varying proportion of non-malignant cell types within tumours or alternative mechanisms of ANKRD11 downregulation in tumour. Since a putative GLI-binding site 5′-GACCgCCCg-3′ was identified within this methylation-sensitive region by *in silico* analysis, we speculated that *ANKRD11* may be a target of GLI1-mediated regulation. To address this possibility, we investigated the potential role of GLI1 in the transcriptional activation of *ANKRD11*.

Moreover, among a set of breast tumours analysed, high levels of *ANKRD11* expression was found in single case of papillary breast cancer, a variant of DCIS where cancer cells are arranged in a finger-like pattern within the ducts (Lim et al., 2012). Experiments were undertaken to investigate the possibility that DCIS, or the papillary variant, is characterised by high expression of *ANKRD11*. Since there is a possible relationship between GLI1 and ANKRD11, gene expression of *GLI1* was also quantified in the DCIS, IBC and normal breast tissue samples.

4.4 Materials and Methods

4.4.1 Clinical sample collections

Tissues were obtained with informed consent from Royal Adelaide Hospital and Victorian Cancer Bank. Ethics approval was obtained from The Royal Adelaide Hospital Research Ethics Committee. Formalin fixed paraffin-embedded tissue was used for immunohistochemistry. Unfixed tissues were stored in RNA*later* solution (Ambion) at -20°C and subsequently used for DNA and RNA extraction using the Allprep DNA/RNA mini kit (Qiagen). Some purified RNA samples (T35-47 and N2.1-2.5) were obtained from Victorian Cancer Bank. Relevant clinical data was retrieved from patient's records including human epidermal growth factor receptor 2 (HER2), estrogen receptor (ER), and progesterone receptor (PR) status.

4.4.2 Plasmids

The luciferase reporter constructs pGL3-*PTCH* and pGL3-*PTCH_mut* (inactive PTCH), and the expression constructs pcDNA4TO empty vector, pcDNA4TO-*GLI1* and pcDNA4TO-*GLI_mut* (inactive GLI1) were kindly provided by Prof. Fritz Aberger from University of Salzburg. The luciferase reporter construct, pGL3-*P5*, has been described in previous report (Lim et al., 2012), and consists of the *ANKRD11* promoter, 5′ UTR and exon 1 (-689 to +306 bp).

4.4.3 Cell cultures and transfection

The human non-malignant immortalized breast epithelial cell line MCF10A, breast cancer cell lines, MDA-MB-231, ZR75-1, MCF-7 and MDA-MB-468, and

embryonic cell line HEK293T were cultured as described (Chapter 3) (Lim et al., 2012). The human pancreatic cell line PANC-1 was kindly provided by Dr. Cara Fraser from Royal Adelaide Hospital. PANC-1 cells were maintained in RPMI supplemented with 10% fetal bovine serum and 100 U/mL penicillin-streptomycin-glutamine (Invitrogen). Expression constructs were transfected into MCF10A cells using Lipofectamine 2000 according to the manufacturer's recommendations.

4.4.4 Reporter Assays

Reporter assays were performed as described (Chapter 3) (Lim et al., 2012). In each well of a 24-well plate, pGL3-*P5*, pGL3-*PTCH*, pGL3-*PTCH_mut* or pGL3-basic vector (0.4 μg) together with the internal control pRL-TK (20 ng) and GLI expression constructs pcDNA4TO-*GLI1*, pcDNA4TO-*GLI1_mut* or pcDNA4TO (10 ng) were co-transfected into the appropriate cell lines in triplicate. Normalised luciferase activity was determined with dual-luciferase reporter assay system (Promega) according to the supplier's recommendations.

4.4.5 Treatment

Demethylating agent 2(1H)-pyrimidinone riboside (zebularine) was purchased from Sigma and dissolved in DMSO. Cells were treated with zebularine for 72 hours with media change every 24 hours to replenish drug levels. Cells were plated at 40% confluence 24 hours prior to the treatment. The approximate doubling time observed using a optical microscope is 24 hours for MDA-MB-231 and MDA-MB-468 cells, 48 hours for MCF-7 and ZR75-1 cells.

4.4.6 RT-qPCR

RNA extraction and RT-qPCR were performed as described (Lim et al., 2012). Relative levels of *GLI1* and *ANKRD11* expression were quantified by RT-qPCR using IQ™ SYBR green supermix (Biorad) and primers listed in Table 4.1. For data analysis, *β-actin* or the nuclear *RPS11* were used as internal reference.

4.4.7 Statistical analysis

Data were analyzed by GraphPad Prism (GraphPad Software, Inc.) using unpaired two-tailed t-tests, Mann-Whitney test and linear and nonlinear regression.

Table 4.1 PCR primers used in this study

Target gene	Forward primer (5'—3')	Reverse primer (5'—3')	Product size (bp)	Location	Annealing temperature, °C
ANKRD11 [NM_013275.4]	AGCCAGGGTGACGAGAACAAGTC	CACACACAGGATCCTCAGTCGTCGTTGACGTCGACCATG	265	Exon 13	57
GLI1 variant 1 [full length GLI1, *NM_005269.2],* 2 [ΔNGLI1*,* NM_001160045.1]*, 3 [tGLI1, NM_001167609.1]*	TCTGGACATACCCCACCTCCCTCTG	ACTGCAGCTCCCCCAATTTTTCTGG	191	Exon 12	59
RPS11 [NM_001015.3]	TGTGTCCATTCGAGGGCGGA	GCACTCGCCCACTGTGACGAT	199	Exon 3-5	57
β-actin [NM_001101.3]	TACCTTCAACTCCATCATGAAGTG	CCGGACTCGTCATACTCCTGCTTG	267	Exon 2	57

ANKRD11: Homo sapiens ankyrin repeat domain 11

GLI1: Homo sapiens glioma-associated oncogene homolog 1 (zinc finger protein)

β-actin: Homo sapiens actin, beta (ACTB)

RPS11: Homo sapiens ribosomal protein S11

4.5 Results

4.5.1 *ANKRD11* and *GLI1* are variably expressed in different grades of invasive breast cancer

Previously, the levels of *ANKRD11* mRNA were assayed in invasive breast tumour tissues and normal samples using RT-qPCR (Chapter 3) (Lim et al., 2012). In the same panel of breast samples, mRNA expression of *GLI1* was analysed. The tumour samples had a lower level of *GLI1* expression (21.5 ± 6.6, n=24) than the normal breast tissues (111.1 ± 59.0, n=4) ($p = 0.003$, Fig. 4.1A and B). Interestingly, papillary DCIS (T14), which previously showed the highest level of *ANKRD11* expression of all the tumours studied (Fig. 3.1) (Lim et al., 2012), also had the highest level of GLI1 expression.

The clinical data showing ER, PR, HER2, MIB-1 status and tumour grade of the breast tumours was reported recently (Table 3.3) (Lim et al., 2012). HER2 negative tumours (38.4 ± 12.8, n = 11) have over a 5-fold higher average level of *GLI1* expression than HER2 positive tumours (7.6 ± 1.1, n = 12; $p < 0.05$) (Fig. 4.1C). Within the IBC samples, the level of *GLI1* message was 5-fold and 13-fold higher in grade 1 tumours (78.2 ± 23.5, n = 4) than grade 2 (16.4 ± 4.6, n = 8; $p < 0.005$) and grade 3 tumours (6.0 ± 1.2, n = 12; $p < 0.0001$), respectively (Fig. 4.1D). Nevertheless, no significant association was found between ER/ PR status and *GLI1* expression, which could be due to the small sample number (Fig. 4.1E and F).

There was a significant correlation between the levels of *GLI1* and *ANKRD11* expression in breast samples (R^2= 0.8, $p< 0.0001$) (Fig. 4.1G). In addition level of

GLI1 expression was not associated with the methylation status of *ANKRD11* (Fig. 4.1H). The data are consistent with the possibility of GLI1 either binding at an alternative location within the *ANKRD11* promoter or indirectly controlling the *ANKRD11* expression. Nevertheless, these findings suggested that *ANKRD11* could be a downstream target of GLI1 and expansion breast tumour panel was needed to gain better insights about the functionality of GLI1.

Figure 4.1

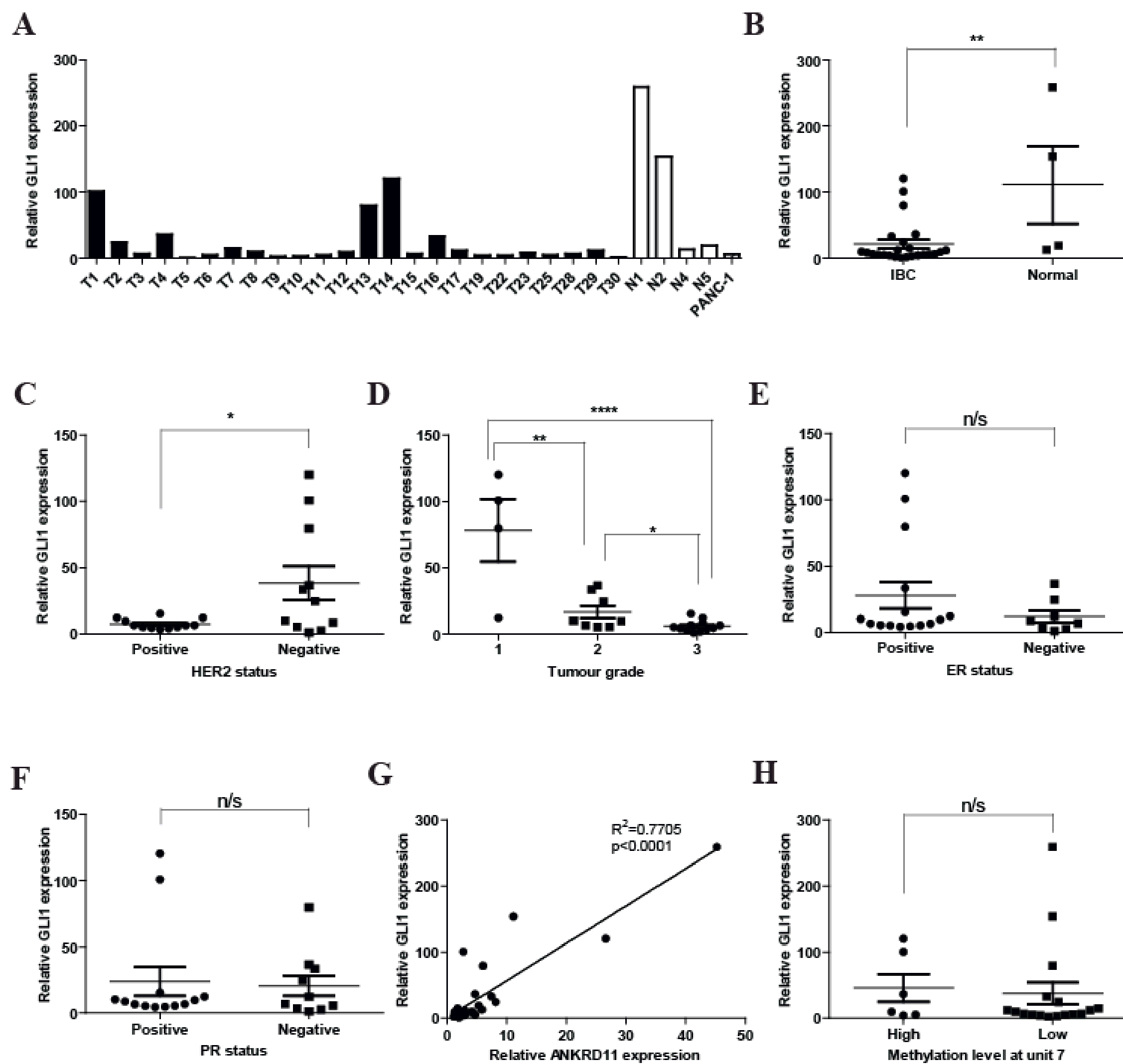


Figure 4.1 The relationship of GLI1 and ANKRD11 expression in invasive breast cancer. (A) The *GLI1* mRNA levels in tumour (T) and normal (N) samples normalised to the housekeeping gene *β-actin*. PANC-1 is a positive control. (B) The distribution of mRNA levels of all tumours (n=24) and normal samples (n=4). The relationship of (C) human epidermal growth factor receptor 2 (HER2) status, (D) tumour grade, (E) estrogen receptor (ER) status and (F) progesterone receptor (PR) status, and *GLI1* mRNA expression in invasive breast tumours. (G) Correlation of relative *ANKRD11* and *GLI1* mRNA levels in human breast tissues. (H) Promoter

methylation of *ANKRD11* promoter at CpG unit 7 (see Chapter 3) and *GLI1* mRNA levels in human breast tissues. High = samples with >50% methylation at unit 7; low = <50% methylation at unit 7. T18, T20, T21, T24, T26 and N3 are not shown due to insufficient samples. An unpaired two-tailed t-test was used for all statistical analysis except (G). The data are presented as mean ± SEM. * = $p < 0.05$. ** = $p < 0.005$. n/s = not significant. The difference between normal and tumour groups claimed in Fig.4.1B remains significance ($p < 0.05$) when non-parametric statistic (Mann-Whitney test) was used. Note the different y axis scales for each panel.

4.5.2 Expression of *GLI1* and *ANKRD11* in different types of breast cancer

A single tumour sample - a papillary breast cancer - had high levels of *ANKRD11* and *GLI1*. To expand the knowledge of the differential expression of *GLI1* and *ANKRD11* in breast cancer, a mixture of breast cancer samples including DCIS, DCIS variant intracystic papillary carcinomas (IPC), micropapillary DCIS (mDCIS) and encapsulated papillary carcinomas (EPC), and tumours containing invasive components were selected (Table 4.2). All of these cancers have excellent prognosis with adequate local therapy (Wynveen et al., 2011). The expression of *GLI1* and *ANKRD11* and clinical data of these samples are listed in Table 4.2.

In the previous study, the levels of *GLI1* and *ANKRD11* mRNA were normalised to the housekeeping gene, *β-actin*, shown in Fig. 4.1. It was possible that the direct relationship between *ANKRD11* and *GLI1* expression may be due to variable expression of this housekeeping gene. To eliminate this possibility, levels of an additional housekeeping gene *RPS11* were assayed by RT-qPCR. *RPS11* was previously identified as a housekeeping gene with very low inter-sample variation based on publicly available microarray data in breast cancer (Popovici et al., 2009). The mRNA expression of *GLI1* was normalised to either *RPS11* or *β-actin* in a panel of breast tissues containing tumour and normal samples. There was a highly significant linear relationship between *RPS11* and *β-actin* normalisation ($p < 0.0001$), confirming the accuracy of the use of *β-actin* as internal control in human breast samples (Fig. 4.2A).

Similar to Fig. 4.1G, a significant relationship between *ANKRD11* and *GLI1* mRNA expression was observed ($p < 0.0001$) (Fig. 4.2B). In addition, higher average relative expression of the *GLI1* mRNA were detected in normal breast tissues from reductions (368.5 ± 153.7, n = 7; $p < 0.0001$) and DCIS (401.7 ± 114.5, n = 8; $p < 0.0001$) compared with breast containing invasive components IBC (9.0 ± 2.7, n = 33) (Fig. 4.2C). A similar pattern was found for *ANKRD11* message where expression of *ANKRD11* were significantly higher in normal breast tissues from reductions (217.6 ± 87.3, n = 7; $p < 0.0001$) and DCIS (338.7 ± 108.5, n = 8; $p < 0.0001$) than IBC (4.1 ± 0.9, n = 33) (Fig. 4.2D). There was an insignificant expression of *GLI1* (0.03 ± 0.006, n = 5) and *ANKRD11* (1.0 ± 0.3, n = 5) in breast cell lines when compared to DCIS and normal breast tissues (Fig. 4.2C and D). Together with previous observation (Fig. 4.1), high expression of *GLI1* and *ANKRD11* appeared to occur in the early stage of breast cancer.

Table 4.2 Clinical data and levels of *ANKRD11* and *GLI1* mRNA in human breast samples

Samples	ER	PR	HER2	Tumour type	Grade	ANKRD11 mRNA	GLI1 mRNA
T31	N/A	N/A	N/A	DCIS	1	7.65	5.20
T32	-	-	-	IDC/ DCIS	2	2.10	0.20
T33	+	+	+++	IDC/ DCIS	3	1.80	0.52
T34	-	+	+++	IDC	2	1.10	0.80
T35	N/A	N/A	N/A	IPC	2	144.80	117.20
T36	+	++	-	mDCIS	1/2	375.20	416.80
T37	N/A	N/A	N/A	mDCIS	1	947.70	818.40
T38	+	-	-	mDCIS	3	215.50	215.80
T39	+	+	-	EPC	2	234.80	581.40
T40	+	++	+++	EPC	2	158.90	195.80
T41	+	+	-	IPC/ EIC/ IC	2/3	1.00	2.80
T42	+	+	-	IPC/ DCIS	1	625.30	863.10
T43	-	-	-	IDC	3	2.80	24.65
T44	-	-	-	IDC	2	20.20	43.31
T45	+	+	-	IDC	2	3.56	6.33
T46	-	-	-	IDC	3	21.86	53.32
T47	+	+	+	IDC	3	9.32	62.39
N2.1	N/A	N/A	N/A	Normal	N/A	407.30	646.57
N2.2	N/A	N/A	N/A	Normal	N/A	227.00	348.09
N2.3	N/A	N/A	N/A	Normal	N/A	639.10	1152.06
N2.4	N/A	N/A	N/A	Normal	N/A	142.00	243.03
N2.5	N/A	N/A	N/A	Normal	N/A	68.10	116.97
N1	*N/A*	*N/A*	*N/A*	*Normal*	*N/A*	*33.10*	*65.30*
N2	*N/A*	*N/A*	*N/A*	*Normal*	*N/A*	*6.80*	*7.80*
T8	*+*	*+*	*-*	*IDC*	*2*	*3.90*	*1.00*

T14	+	+	-	*IPC/ IC*	*1*	*9.31*	*4.30*
PANC-1						*2.30*	*0.88*

ER, PR, HER2, MIB-1 count, tumour type and grades of each tumour sample are shown.

N1, N2, T8 and T14 are previously shown in Fig. 4.1. The relative mRNA expression of *GLI1* and *ANKRD11* were normalised to housekeeping gene *β-actin*. PANC-1 is a pancreatic cell line, which is known to contain high level of *GLI1* mRNA (Thayer et al., 2003). N/A: not available; DCIS: Ductal Carcinoma *In situ*; mDCIS: Micropapillary DCIS; IDC: Invasive Ductal Carcinoma; IPC: Intracystic Papillary Carcinoma; IC: Invasive Carcinoma; EPC: Encapsulated Papillary Carcinoma; EIC: Extensive Intraductal Carcinoma; Normal: normal breast tissues from breast reductions.

Figure 4.2

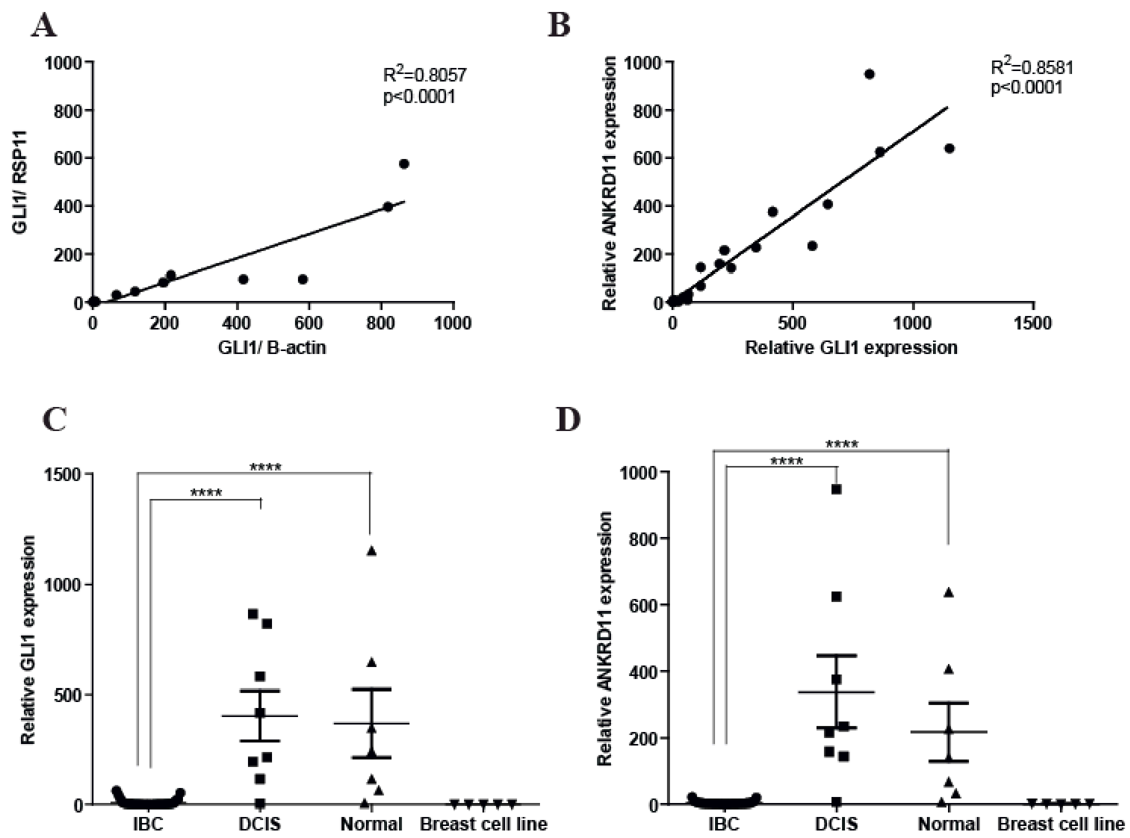


Figure 4.2 The expression of *GLI1* and *ANKRD11* in DCIS and IBC. (A) The use of the housekeeping gene, *β-actin*, as an internal control for relative *GLI1* expression was validated using *RPS11*. (B) The linear relationship of *GLI1* and *ANKRD11* mRNA expression in human breast tissues as described in Table 4.2. Comparison of the relative expression of (C) *GLI1* and (D) *ANKRD11* in different types of breast tissues as described in Table 4.2 and Fig. 4.1. Tumours containing invasive components are categorised as IBC. DCIS and its variant IPC/EPC are combined as DCIS. Information of breast cell lines can be found in Fig. 4.3. An unpaired two-tailed t-test was used for statistical analysis in (C) and (D). The data are presented as mean ± SEM. **** = p < 0.0001.

4.5.3 *GLI1* and *ANKRD11* mRNA expression in breast cell lines

The levels of *GLI1* and *ANKRD11* expression in five human breast cell lines and human pancreatic cell line PANC-1 were examined by RT-qPCR (Fig. 4.3). PANC-1 reported to have high levels of GLI1 was used as a positive control (Nolan-Stevaux et al., 2009, Thayer et al., 2003). The result showed that the expression of *GLI1* in PANC-1 cells is 30-fold higher than the five breast cell lines, while the levels of *ANKRD11* in PANC-1 cells were similar to the breast cell lines (Fig. 4.3A). Notably, breast cell lines with relatively high *GLI1* expression also have high level of *ANKRD11* expression (Fig. 4.3A).

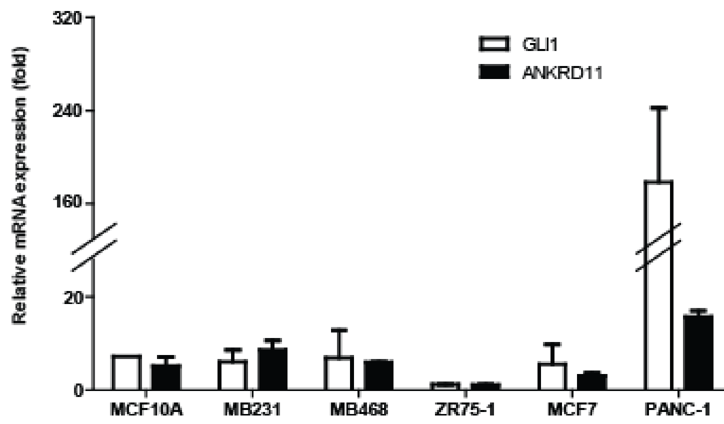
A previous study showed levels of *GLI1* mRNA in breast cell lines as MCF10A ≈ MDA-MB-231> MDA-MB-468> ZR75-1> MCF-7 (ten Haaf et al., 2009). Breast cancer cell lines MDA-MB-231 (ER-) and MDA-MB-468 (ER-), and non-tumorigenic breast cell line MCF10A (ER-) exhibit myoepithelial characteristics are known to have higher invasive capacity than MCF-7 (ER+) and ZR75-1(ER+), which have pure luminal phenotype (Gordon et al., 2003). In this study, consistent higher levels of GLI1 expression were found in MCF10A, MDA-MB-231 and MDA-MB-468 compared with MCF-7 and ZR75-1 (Fig. 4.3). However, mRNA levels of *GLI1* in these breast cells were negligible when compared to the human breast tissue samples (Fig. 4.2).

MCF10A cells were transiently-transfected with the GLI1-expression construct to study the expression of *ANKRD11* in the presence of GLI1 overexpression. GLI1 ectopic expression modestly upregulated the endogenous levels of *ANKRD11* expression by 50% in MCF10A cells (Fig. 4.3B). The levels of

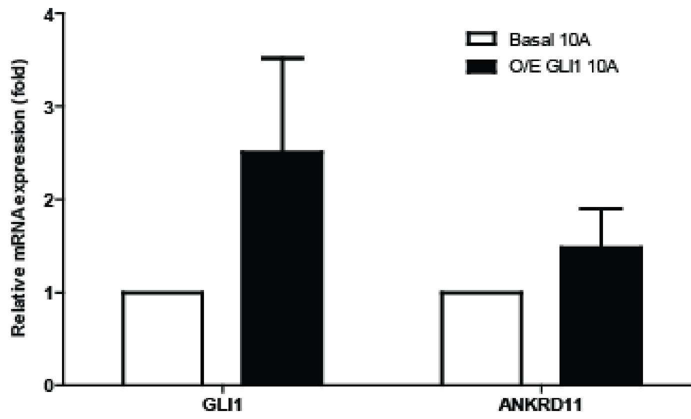
ANKRD11 expression are upregulated in ZR75-1, but not MCF-7 cells, following treatment with the demethylating agent zebularine (Chapter 3) (Lim et al., 2012). However, in this case no corresponding increase in *GLI1* expression was observed, suggesting increased *ANKRD11* expression did not affect the endogenous expression of *GLI1* (Fig. 4.3C). Collectively, the data is consistent with the control of *ANKRD11* expression directly or indirectly by GLI1, whereas ANKRD11 is unlikely to have a role in regulating *GLI1* expression.

Figure 4.3

A



B



C

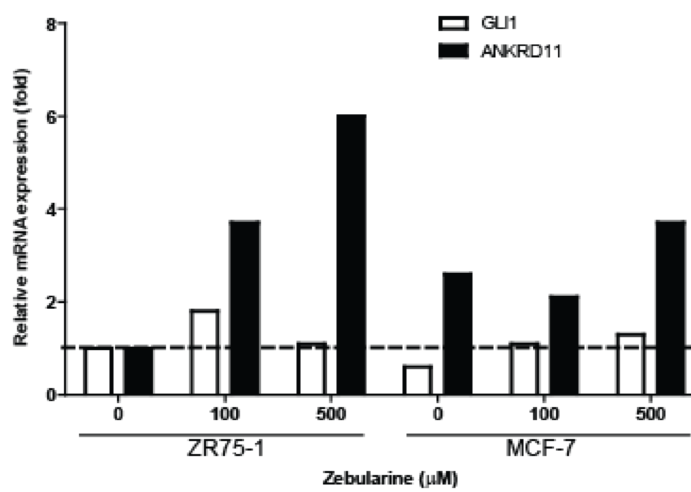


Figure 4.3 The relationship of *ANKRD11* and *GLI1* in breast cell lines. (A) The relative levels of *GLI1* and *ANKRD11* expression were assayed in breast cancer cell lines (n = 3) and pancreatic cell line PANC-1 (n = 2) (positive control). The data are presented as mean ± SEM. (B) The relative expression of *GLI1* and *ANKRD11* were quantified in immortalised normal breast cell line MCF10A overexpressing GLI1 (n=3). The expression levels relative to the basal expression in MCF10A cells are shown. (C) *GLI1* and *ANKRD11* mRNA levels are shown relative to untreated ZR75-1 and MCF-7 cells. The cells were treated with indicated concentrations of zebularine for 72 hours. The relative mRNA expression of *ANKRD11* and *GLI1* were normalised to *β-actin*. Note the different y axis scales for each panel.

4.5.4 The *ANKRD11* promoter region between -689 and +306 bp may not be the binding site for GLI1

Previously we determined methylation of a 19 bp region between -582 to -574 bp of the *ANKRD11* promoter was related to the expression levels of *ANKRD11* in breast cell lines (Chapter 3). This methylated region contains a putative GLI1 binding site. To determine whether GLI1 can directly transactivate this region of the *ANKRD11* promoter, a luciferase reporter construct was generated where the luciferase gene is under the transcriptional control of the human *ANKRD11* promoter (-689 to +306 bp).

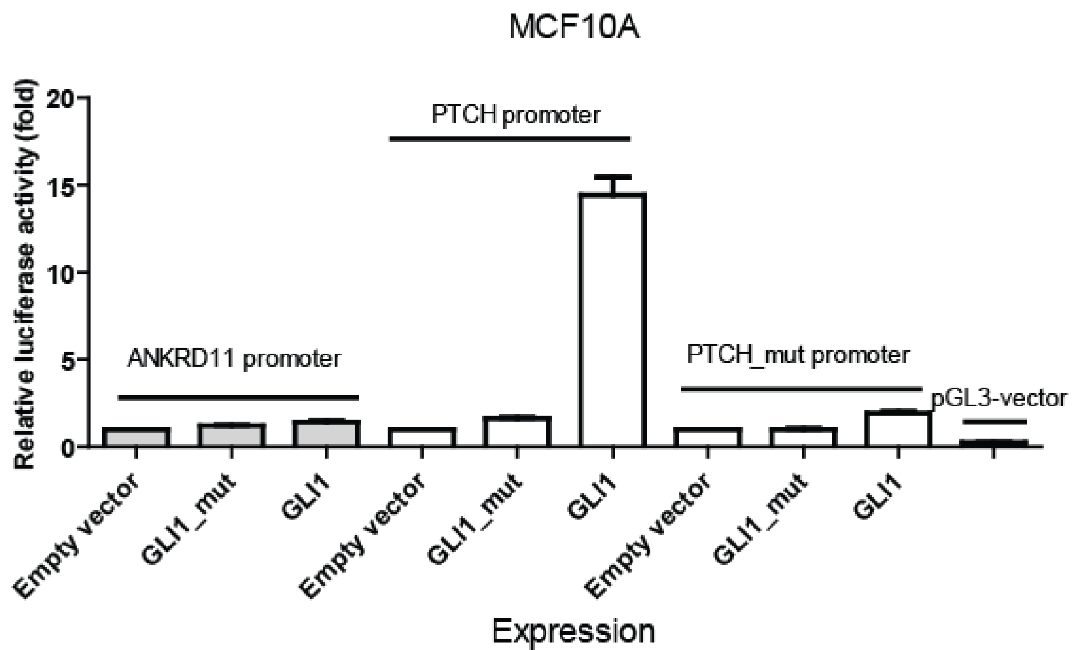
A luciferase construct containing the *PTCH* promoter was used as a positive control. *PTCH* is a known direct target of GLI1 (Regl et al., 2004) and is upregulated in the presence of increased GLI1 expression (see Fig. 4.4). A higher level of transcriptional activity of the PTCH promoter was seen in MCF10A cells compared with HEK293T cells (Fig 4.4A and B). A control construct (PTCH_mut) is included where a single base substitution in the PTCH promoter within the GLI1 DNA binding site completely abrogates its transcriptional activity (Winklmayr et al., 2010).

To examine if GLI1 influences transcriptional activity of *ANKRD11*, expression constructs expressing GLI1 or GLI1_mut proteins were co-transfected with a *ANKRD11* reporter construct into MCF10A and HEK293T cells. Specific increase in PTCH promoter activity in response to exogenous GLI1, but not GLI1_mut expression, confirmed the validity of the reporter system. However, no significant difference was found in the *ANKRD11* promoter activity in the GLI1-expressing cells in both cell lines. This suggested that GLI1 is not likely to influence

the transcriptional activity of *ANKRD11* at this specific region of the promoter, -689 to +306 bp (Fig. 4.4A and B).

Figure 4.4

A



B

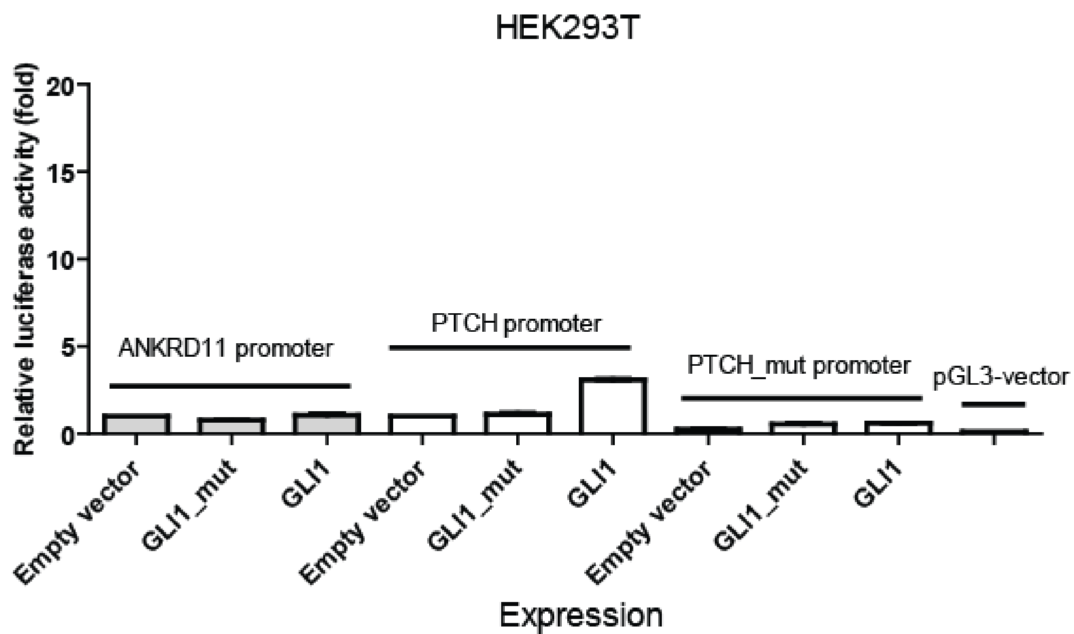


Figure 4.4 The transcriptional activity of *ANKRD11*, *PTCH* (positive control) and *PTCH_mut* (negative control) were assayed in (A) MCF10A and (B) HEK293T cells over-expressing GLI1 or GLI1_mut (negative control) proteins. The promoter activity

of *ANKRD11*, *PTCH* and *PTCH_mut* in cells overexpressed with GLI1 or GLI1_mut was normalised to the promoter activity in cells overexpressed with empty vector (pcDNA4TO vector). GLI1_mut was used as a negative control for GLI1 expression as there is mutation in its DNA binding domain and thus it does not bind to the promoter region of *PTCH*. Values represent averages of triplicate treatments.

4.6 Discussion

We have previously reported ANKRD11 as a putative tumour suppressor and p53-coactivator in breast cancer (Neilsen et al., 2008). In Chapter 3, a putative GLI1-binding site located within a methylation-sensitive region of the *ANKRD11* promoter was identified using *in silico* analysis (Lim et al., 2012). By the use of dual luciferase reporter assays, there was no evidence for direct transcriptional regulation of the region of *ANKRD11* promoter containing the putative GLI1-binding site.

In addition, these experiments were designed to investigate the relationship between *GLI1* and *ANKRD11* expression, and in particular, whether papillary breast cancer (a variant of DCIS, ductal carcinoma *in situ*) is associated with high *ANKRD11* expression. The results indicated that *GLI1* and *ANKRD11* were both expressed at higher levels in various subtypes of papillary carcinoma but also in classical DCIS. However, the functional role of these changes in expression are presently unknown.

As a linear relationship was observed in the levels of *GLI1* and *ANKRD11* expression (Fig. 4.1 and 4.2), it was speculated that *ANKRD11* is directly or indirectly regulated by the GLI1 transcription factor. Indeed GLI1-overexpression modestly upregulated endogenous *ANKRD11* expression in MCF10A cells. However, despite the fact that no relationship was seen between GLI1 and ANKRD11 with promoter luciferase assays, these assays were limited to a restricted region of the *ANKRD11* promoter. It is possible that a GLI1 response element may be located at other regions of the *ANKRD11* promoter. Therefore, further studies are warranted to investigate the possibility that *ANKRD11* expression is directly controlled by GLI1. It is also likely

that *GLI1* may influence *ANKRD11* transcriptional activity indirectly via a presently unknown transcription factor.

Recent studies revealed distinct patterns of expression of two proteins, p53 and SMO, in IBC (Stecca and Ruiz i Altaba, 2009). SMO is a key component of Hh signalling pathway that mediates signals from the surface of cells to downstream pathway components such as GLI1 (Wang et al., 2009). The tumour suppressor p53 is known to repress GLI1 in the Hh signalling pathway (Stecca and Ruiz i Altaba, 2009). In breast cancer, the frequency of p53 overexpression appears to be related to the pathological stage (Lane, 1992). The mutant p53 protein is overexpressed in 50% of invasive tumours and 15% of non-invasive tumours (Davidoff et al., 1991). The majority of our IBC samples possessed a lower level of *GLI1* expression (Fig. 4.1 and Fig. 4.2) and this maybe related to the expected high levels of p53 protein.

GLI1 is a nuclear protein (Kinzler and Vogelstein, 1990). In the Hh signalling pathway, SMO is known as the sole effector of GLI1 that responsible for releasing GLI1 from cytoskeleton inhibition (Jia et al., 2003). As a result, it is possible that the level of SMO reflects the amount of activated GLI1. A previous study has shown that SMO is highly expressed in ~70% of DCIS and only expressed in ~30% of IBC samples (Moraes et al., 2007). Conflictingly, the percentage of GLI1 nuclear translocalisation in IBC was significantly higher than DCIS (Souzaki et al., 2011), showing that the SMO expression may not dictate the amount of activated GLI1 in the nucleus. However, this is consistent with the role of GLI1 protein in upregulating MMP-11, which is associated with assisting cancer cells to damage the basement membrane and invade the capillary that often occurs in IBC (Kwon et al., 2011).

Nevertheless, we have found that *GLI1* mRNA is highly expressed in most of our DCIS samples and lowly expressed in IBC samples.

Previous immunohistochemistry-based study showed association of ER status, but not HER2 status, with GLI1 expression (Xu et al., 2010). In contrast, we have found a significant association of mRNA level of *GLI1* with HER2 status but not with ER status or PR status. A number of factors could explain this contradictory observations. It is known that GLI1 protein is functional when translocated to the nucleus (Kinzler and Vogelstein, 1990). Since the immunohistochemistry studies scored nuclear signal and not total levels of cellular GLI1 protein, this may contribute to the apparent lack of correlation between mRNA and protein levels. Collectively, we hypothesise that elimination of *GLI1* mRNA expression, and elevation of GLI1 protein expression, may be required for the malignant transformation of DCIS or papillary carcinoma to invasive breast cancer.

A number of experiments may be undertaken in the future to establish the molecular basis of the novel finding of a linear relationship between *GLI1* and *ANKRD11* mRNA expression. Our result suggest that GLI1 does not bind to the critical methylation-specific region of the *ANKRD11* promoter. Chromatin immunoprecipitation (ChIP) assays will be essential to complement luciferase based reporter assays to confirm if there are GLI1 binding sites elsewhere in the *ANKRD11* promoter. To relate the levels of *GLI1* and *ANKRD11* message to functional protein immunohistochemistry of DCIS and IDC are required. It should be noted that function of GLI1 protein in the nucleus is known to be essential for Hh signalling pathway (Kinzler and Vogelstein, 1990). However, the *GLI1* message that was assessed by

RT-qPCR will include message in both cytoplasm and nucleus. Confirmation of GLI1 protein localisation will be required to confirm the role of *GLI1* mRNA in human breast cancer.

In conclusion, ANKRD11 may have a direct relationship with GLI1 although further studies are required to confirm this. *ANKRD11* and *GLI1* mRNA are highly expressed in normal breast tissues and less invasive type of breast cancer. Further functional analyses such as those outlined here will be required to investigate the underlying mechanisms of ANKRD11 and GLI1 expression and relevance in invasive breast cancer samples.

4.7 Acknowledgements

Plasmids, pcDNA4TO-GLI1, pcDNA4TO-GLI1_mut, pcDNA4TO, pGL3-basic-PTCH and pGL3-basic-PTCH_mut, were kindly provided by Prof. Fritz Aberger, University of Salzburg, Austria. PANC-1 cell line was donated by Dr. Cara Fraser, Royal Adelaide Hospital, South Australia.

GENERAL DISCUSSION

The unifying theme of this thesis is the role of epigenetics in cancer. There are two major aims, the first to develop a high throughput test, EPISSAY, to determine the relative activities of compounds that can reverse epigenetic changes. This first aim included Chapter 1, a review paper published as “The application of delivery systems for DNA methyltransferase inhibitors” in *Biodrugs* and Chapter 2, a submitted paper entitled “Development of a novel cell-based assay system EPISSAY for screening epigenetic drugs and liposomes formulated decitabine”. Since cancer is associated with hypermethylation of gene promoters of tumour suppressor genes, such compounds are potential anticancer agents (Appleton et al., 2007, Pohlmann et al., 2002, Auerkari, 2006, Zambrano et al., 2005). Demethylation agents are a major class of epigenetic based drugs and decitabine is now used in the clinic. The EPISSAY system was successfully developed and implemented and was used to compare the activity of a variety of epigenetic-based compounds.

The development of epigenetic drugs with better bioavailability and potency has been hampered due to the difficulties in assessing the efficiencies of newly developed or modified epigenetic drugs (Lim et al., 2011). Currently used assay systems for epigenetic drugs are limited to the quantification of the re-expression of known epigenetically-silenced genes by RT-qPCR and western blot analysis or estimation of global DNA methylation using capillary electrophoresis, DNA digestion with methylation-sensitive restriction enzymes, or analysis of specific DNA

methylation using bisulfite sequencing and methylation-specific PCR (Byun et al., 2008, Butler et al., 2002, Villar-Garea et al., 2003). However, these assay systems are time-consuming, cumbersome and subject to misinterpretation (Biard et al., 1992, Okochi-Takada et al., 2004, Hassig et al., 2008). To tackle these issues, a cell-based assay system, termed 'EPISSAY', was developed to assay for reactivation of an epigenetically silenced gene using flow cytometry. The EPISSAY system uses RFP as a readout for gene reactivation enabling a high throughput format and is therefore advantageous for high throughput screening purposes.

A significant finding of the function of epigenetic drugs was that the reactivation of gene expression induced by treatment with decitabine or zebularine was not necessarily caused by DNA demethylation (Chapter 2). Following methylation based SEQUENOM MassARRAY EpiTYPER analysis, it was consistently found that gene reactivation in EPISSAY cells after treatment with decitabine or zebularine was not related to the methylation status of the CMV promoter. Therefore, the EPISSAY system is likely to be based on gene reactivation not by demethylation, but by alteration of chromatin tertiary structure, probably by alterations in histone modifications such as the histone mark H3K4me3 (Cedar and Bergman, 2009, Thomson et al., 2010, Li et al., 2011, Ke et al., 2010). This is consistent with the reports that showed decitabine or zebularine can also reactivate histone-induced silenced genes (Halaban et al., 2009, Scott et al., 2007, Radhakrishnan et al., 2008, Savickiene et al., 2012, Lavelle et al., 2006). It is also possible that the gene reactivation observed was induced by cellular stress response (Sutton et al., 2002, Tsai et al., 2012).

In clinical trials in patients with solid tumours and blood-related disease such as MDS, there was no significant relationship between clinical response and the demethylation of known hypermethylated genes such as p15/INK4B (Daskalakis et al., 2002, Stewart et al., 2009, Appleton et al., 2007, Issa et al., 2004). Another clinical study showed that the combination treatment of decitabine with HDAC inhibitors, such as valproic acid, improved clinical response in MDS patients (Garcia-Manero et al., 2006). In these studies there was no relationship between clinical response and the induction of histone acetylation by the HDAC inhibitor (Garcia-Manero et al., 2006). These studies suggested that direct modifications of epigenetic mechanism are not the prerequisites for the clinical response observed.

A recent study in patients with acute myeloid leukemia showed elevated expression of miR-29b targeting DNMTs and receptor tyrosine kinases was found to be associated with clinical response after a 10-day decitabine administration (Blum et al., 2012). In addition, recent analysis revealed that the expression of a few genes was uniquely altered by the combination treatment of decitabine and the HDAC inhibitor panobinostat in diffuse large B-cell lymphoma (DLBCL) cells (Kalac et al., 2011). These genes were *VHL*, *TCEB1*, *WT1* and *DIRAS3*, which are all involved in controlling cell proliferation (Makki et al., 2008, Zou et al., 2011, van Hagen et al., 2009, Kamura et al., 1999). The expression of these genes played a major part in inhibiting growth and initiating the apoptosis observed in DLBCL cells after treatment with decitabine and panobinostat (Kalac et al., 2011). These findings supported a different aspect of the *in vivo* functions of these drugs in changing the expression of particular genes to achieve unique profiles for certain pathways, and also confirmed that the most important role of an assay system for epigenetic drugs

was to examine the level of gene expression. Although the *in vivo* mechanisms involved in epigenetic-based drugs are still to be resolved, they are being used increasing in the clinic. It is likely that the critical gene targets *in vivo* that are reactivated by epigenetic agents have yet to be determined, however, could play a major role in determining clinical outcomes.

DNA methylation is critical to modulate the readout of the genome as the correct timing and expression of information can dictate the generation of different cell types in development (Bird, 2002, Jones and Baylin, 2002, Herman and Baylin, 2003, Novak et al., 2008). Since the 1970s, demethylating agents, such as decitabine and zebularine, have been synthesised to reverse DNA hypermethylation in cancer and so reactivate tumour suppressor genes (Cihak, 1974, Li et al., 1970). At low doses, the functions of such agents are to reactivate expression of genes that are responsible to induce cell proliferation, apoptosis and cell death (Lyko and Brown, 2005, Gottlicher et al., 2001, Singh et al., 2005, Baylin, 2005), and to increase sensitivity of cancer cells to chemotherapeutic agent such as cisplatin (Teodoridis et al., 2004). At high doses, these drugs have been used as direct cytotoxic agents that causes death of rapidly dividing cancer cells (Kantarjian and Issa, 2005).

Although decitabine is the most potent demethylating agent available, it has limited activity on solid tumours (Lim et al., 2011). Both *in vitro* and *in vivo*, decitabine is prone to undergo hydrolysis (Beisler, 1978) and degradation by cytidine deaminase (Chabot et al., 1983), resulting in conversion to an inactive form. To improve the stability of decitabine, nano-encapsulation of the clinically used drug

decitabine was developed. The formulated decitabine with improved potency was demonstrated in the EPISSAY system.

Liposomes contain artificial biomembranes that protect inner vesicles from environmental stresses and allow for the controlled release of encapsulated constituents (Sessa and Weissmann, 1968, Smith et al., 2010, Pannier and Shea, 2004). Liposomes have been FDA approved in carrying Doxorubin (DOXIL/Caelyx) for treating ovarian cancer (Rose, 2005). In this study, multilamellar liposomal decitabine was formulated (~1000 nm). The liposomes were extruded in a specific membrane to gain unilamellar liposomes with a size of ~150 nm. The unilamellar form of this newly formulated decitabine was found to be 50% more potent than the multilamellar liposomal decitabine or the pure drug when assayed using the EPISSAY system. These results were supported by a controlled release study comparing the drug release of decitabine from unilamellar and multilamellar liposomes. This showed that the release rate of decitabine from unilamellar liposomes was slower, suggesting unilamellar liposomal formulation may decrease the rate of degradation of decitabine by providing protection to the drug. Using the 7-AAD cytotoxicity assay, the liposomal decitabine tested showed a similar toxicity to the pure decitabine, confirming the safety of the liposomal formulation. As the surface of the liposomal decitabine was functionalised with a PEG polymer, the *in vivo* half-life the drug will have improved (Veronese and Pasut, 2005).

In addition to the chemically instability, another major problem surrounding decitabine use is its transient property (Kagey et al., 2010, McGarvey et al., 2006). This was showed *in vitro* where withdrawal of decitabine re-silences previously

demethylated genes by nucleosome occupancy and active histone modification of H3K9ac (Si et al., 2010). This could be an explanation for the complexity of decitabine treatment regime that requires continuous administration to achieve a clinical response (Samlowski et al., 2005, O. Odenike, 2007). Recent study showed that low-dose decitabine resulted in longer anti-tumour effect and high dose was cytotoxic (Tsai et al., 2012). Our formulation may overcome the transient effects of decitabine by providing a continuous and possible low-dose release of the drug using unilamellar liposomes.

Decitabine also has limited activity on solid tumours (Appleton et al., 2007, Stewart et al., 2009, Cowan et al., 2010). Fortunately, the liposomal formulation of decitabine has achieved a size of ~150 nm. As nanoparticles (< 250 nm) tend to accumulate in the disease tissues that undergo rapid angiogenesis via the EPR effect (Iyer et al., 2006), together with the protective nature of the liposomes, a sufficient amount of decitabine may be targeted to the solid tumours. Additionally, the surface properties of liposomes allow additional further modification of the liposomal decitabine to be undertaken (Kircheis et al., 2001, Faraasen et al., 2003). Future work will be focused toward identifying specific ligands that can specifically target the breast tumours and subsequently minimise the non-specific effect of decitabine (Hamm et al., 2009, Kircheis et al., 2001).

Collectively, the liposomal decitabine that was synthesised here was validated as a potential safe and more potent epigenetic drug with better potential in clinical application. However, we have only confirmed this *in vitro*. An *in vivo* study of liposomal decitabine is recommended to assess its applicability for clinical use, and to

confirm if the present limitations of decitabine use in the clinic could be overcome by this formulation.

The second aim centred on the gene *ANKRD11*, which has previously characterised as a potential breast tumour suppressor gene in our laboratory. In this study, expression and methylation patterns of *ANKRD11* were determined in breast tumour cell lines and normal breast tissues, DCIS and IBC, and the relationship between expression of the transcription factor GLI1 and *ANKRD11* in contributing to breast cancer was explored. This aim included Chapter 3, published as "Specific-site methylation of tumour suppressor ANKRD11 in breast cancer" in *European Journal of Cancer* and Chapter 4, a paper in preparation entitled "Differential expression of ANKRD11 and GLI1 in ductal carcinoma *in situ* and invasive carcinoma of the breast".

ANKRD11 was described as a p53-coactivator and putative tumour suppressor in our laboratory (Neilsen et al., 2008). However, the mechanism causing the gene silencing of *ANKRD11* was not previously studied. In this thesis, the mRNA level and DNA methylation of *ANKRD11* were analysed in breast cancer samples. When compared to normal breast tissues, *ANKRD11* mRNA in breast tumours was uniformly downregulated except in one case of papillary breast cancer (Chapter 3). Papillary breast cancer, which is associated with a good prognosis, is a variant of DCIS where cancer cells are arranged in a finger-like pattern within the breast ductal tissue. Further investigation revealed that papillary breast cancer was characterised by a high expression of *ANKRD11*. However, analysis of additional classical DCIS

samples suggest high levels of *ANKRD11* are a characteristic of DCIS and may not limited to the papillary subtype.

In order to study DNA methylation of *ANKRD11*, promoter analysis was carried out by luciferase assays (Lim et al., 2012). In this study, a critical promoter region of *ANKRD11* was identified at a CpG-rich fragment between -661 and -571 bp located in the 5′ UTR. Within this region, a significantly higher level of methylation was found in a CpG unit containing three CpGs (-582 to -574 bp) in the breast tumour tissue samples compared with normal samples using the SEQUENOM EpiTYPER platform. In cells treated with the demethylating agent zebularine, demethylation was also found in the same region of the *ANKRD11* promoter and upregulation of *ANKRD11* was observed, suggesting that this CpG unit is an important region for controlling gene expression.

In a total of 25 IBC type of tumour samples, 36% of tumours had a high methylation level (>50%), 24% of tumours had a moderate level of methylation (15-50%), 40% of tumours had a similar level of methylation (<15%) as the normal blood and tissue samples. These results suggested that the downregulation of *ANKRD11* in 36 to 60% tumours is related to the increased methylation of the *ANKRD11* promoter at the critical CpG unit, whereas gene silencing of *ANKRD11* for the rest of the tumours is due to other mechanisms. Interestingly, the sole case of papillary breast cancer examined, which had a high mRNA level of *ANKRD11*, also had a high level of ANKRD11 methylation (86%), suggesting that the expression of *ANKRD11* is not regulated by methylation in this particular type of breast cancer. As this was the only case of papillary type of DCIS intermixed with an invasive breast cancer component

in the tumour panel, it is possible that the upregulation of *ANKRD11* is controlled by another mechanism that is not present in other IBCs. However, an additional methylation study on a panel of papillary breast cancer is required to examine this in more detail.

Genes displaying a homogenous methylation pattern at a highly defined region and with minimal background in normal tissue are used as diagnostic tools for cancer, such as BRCA1, p16/CDKN2A and RASSF1A methylation (Herceg and Vaissière, 2011). Currently clinical used epigenetic biomarkers are SEPT9, VIM, SHOX2, GSTP1 and MGMT (Heichman and Warren, 2012). Of interest, the specific methylation state of the tumour suppressor gene *ANKRD11* in tumour samples was shown to correlate with HER2 status. HER2-status is often used to predict clinical outcome and response to hormone therapy (Carney et al., 2007). HER2-negative breast cancer is less aggressive than HER2-positive breast cancer and with better prognosis (Beltràn and Colomer, 2002). In our study, a significant relationship was found between HER2-negative tumours and high level specific-site methylation of *ANKRD11*. These findings may imply a potential of ANKRD11 methylation as a biomarker for prognosis or for therapy recommendation in breast cancer.

In silico analysis revealed a putative GLI1 binding site (Ji et al., 2006, Winklmayr et al., 2010) on the specific methylation region of the *ANKRD11* promoter, suggesting that the expression of *ANKRD11* may be regulated by GLI1. The study was done by using a luciferase reporter under the transcriptional control of the human *ANKRD11* promoter between -689 and +306 bp that contained the methylation-sensitive region. However, transcriptional activity of *ANKRD11* in the

cells overexpressed with GLI1 was not affected. In addition, no relationship was found between specific-site methylation of the *ANKRD11* promoter and endogenous mRNA levels of *GLI1*, suggesting that *ANKRD11* may not be directly regulated by GLI1 at the specific methylated region. Nevertheless, endogenous *ANKRD11* expression was upregulated in cells overexpressed with GLI1. Collectively, no obvious evidence has been found that *ANKRD11* is regulated by GLI1 at this specific region. Nevertheless, we could not disregard a possibility that other transcription factor bind to this specific region of the *ANKRD11* promoter where further investigation is required using ChIP assay. In addition, it is also possible that GLI1-binding site is located at the other regions of the *ANKRD11* promoter.

In contrast to the tumour suppressor gene *ANKRD11*, *GLI1* has a role in driving invasion and migration in breast cancer (Kwon et al., 2011, Katoh and Katoh, 2009). Regardless of their opposite roles, a linear relationship was revealed between the mRNA level *ANKRD11* and *GLI1* in human breast tissues (Chapter 4). Indeed the expression patterns of these two genes are remarkably similar. As a tumour suppressor gene, *ANKRD11* is highly expressed in low tumour grade breast tissues, suggesting the expression of *ANKRD11* may contribute to the good prognosis of these tumours. Our results support the role of *GLI1* by showing that *GLI1* is overexpressed in DCIS where an invasion factor is required to progress the cancer to a more aggressive state. However, the mRNA expression of GLI1 that is reported here did not correlate with the level of GLI1 nuclear localisation in DCIS reported in a previous study (Souzaki et al., 2011). Further immunohistochemistry and functional analyses are required to confirm the biological relationships of ANKRD11 and GLI1 proteins.

FINAL CONCLUSION

In this thesis, a standard cell-based assay system and a liposomal formulation that could improve the epigenetic based-therapeutic approach were developed. Although the cell-based assay system was not DNA methylation-specific, it is robust and was successfully used to assay existing and newly formulated epigenetic drugs for gene reactivation. A potent and safe unilamellar liposomal decitabine was formulated and tested using this system.

ANKRD11 is known as a tumour suppressor gene (Lim et al., 2012) and *GLI1* is known as a oncogene (Fiaschi et al., 2009). Available evidence suggests these seem to be playing independent roles in breast cancer. However, in the second part of this thesis, we have discovered an elevated expression of *ANKRD11* and *GLI1* in DCIS, a pre-invasive form of breast cancer.

Moreover, a significantly high level of specific-site DNA methylation of the *ANKRD11* promoter was found in breast tumour samples, especially in HER2 negative tumours, where gene silencing of *ANKRD11* could be partially due to this DNA methylation in breast cancer.

In conclusion, the findings here offer to improve the epigenetic cancer therapeutics and has identified a novel relationship between genes that may provide clinical markers for breast cancer prognosis.

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