Effects of Cisplatin and Interference Peptides on Triple Negative Breast Cancers

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PREFACE

This thesis was supervised by Associate Professor Pilar Blancafort (School of Anatomy, Physiology and Human Biology, UWA), Assistant Professor Foteini Hassiotou (School of Chemistry and Biochemistry, UWA) and Professor Luis Filgueira (Department of Medicine, University of Fribourg, Switzerland). My candidature was sponsored by the Malaysian Ministry of Higher Education (MOHE). The work presented in this thesis is my own work except when stated. It was primarily carried out in School of Anatomy, Physiology and Human Biology in the Faculty of Science, UWA.

The material presented in this thesis has not been presented for any other degree. One chapter of this thesis has been published (Chapter 3). This publication is outlined in the *Statement of Candidature Contribution*. The work of this thesis has also been presented at scientific conferences in both oral and poster formats, as outlined in *Publications*. 
ABSTRACT

Triple negative breast cancers (TNBCs) are very aggressive cancers with poor prognosis and very low survival rates, known to be enriched in cancer stem cells (CSCs). CSCs within breast tumours are associated with cell proliferation and metastasis, and a less differentiated tumour phenotype. Previous studies have shown that tumours enriched in CSCs are sensitive to platinum-based anti-cancer drugs, such as cisplatin. The primary aim of this thesis was to examine the anti-cancer effects of cisplatin in TNBCs as a single drug as well as in combination with two new targeted therapies, the Engrailed-1 (EN1) and SOX2 interfering peptides (iPeps), with each targeting a different transcription factor (TF).

In the third chapter, the potential of cisplatin to induce differentiation was examined in breast cancer cell lines that represent different breast cancer subtypes. BT-549, MDA-MB-231 and MDA-MB-468 TNBC cell lines along with estrogen and progesterone receptor positive MCF-7 cells were tested. Cisplatin treatment of 10 µM and 20 µM reduced cell viability by 36-51% and proliferation capacity by 36-67%. This also resulted in 12-67% down-regulation of stem cell markers (CD49f, SSEA4) and 10-130% up-regulation of differentiation markers (CK18, SMA, β-tubulin), demonstrating a shift in the cellular hierarchy of the tumour towards more differentiated cells. At the mRNA level, CD49f was down-regulated, whilst β-tubulin was up-regulated in the claudin-low cell lines, in accordance with the protein data. However, SSEA4 mRNA expression increased, in contrast to the protein levels of this marker which decreased, suggesting differential regulation of cisplatin at the post-transcriptional level. The reduction in breast cancer cell survival and induction of cellular differentiation upon cisplatin treatment provided evidence on the potential of cisplatin to target specific chemotherapy-resistant cells within a tumour.

In Chapter 4, I investigated targeted inhibition of a neural-specific TF, Engrailed 1 (EN1), which has been recently shown by our group to be overexpressed in inflammatory breast cancer (IBC). An interference peptide (iPep) was used to specifically target EN1 and examine its mechanism of action on this TF. Treatment of SUM-149 breast cancer cells with an active EN1 iPep for 8 hours resulted in 50% reduction of cell viability (IC\textsubscript{50}) at 10.1 µM, while a mutant EN1 iPep had no effect on the cells. In comparison, cisplatin treatment of SUM-149 cells resulted in reduction of cell viability with an IC\textsubscript{50} of 18.35 µM. Interestingly, the active iPep did not synergize
with cisplatin in a combinatorial treatment; instead, the iPep suppressed the effect of cisplatin. The two very different mechanisms and pathways of action of the iPep and cisplatin could have prevented a significant synergistic interaction between the drugs. The EN1 iPep showed a co-localization with gluthamyl-prolyl tRNA (EPRS), an important protein involved in the translational control of inflammatory agents. EPRS is a tRNA synthetase which catalyses the ligation of glutamine and proline to the cognate tRNAs respectively. The binding of the iPep to the active pocket of EPRS is also the active proline binding site. Hence the ability of the iPep to block catalysis of proline by EPRS was examined using a proline incorporation assay. As expected, proline incorporation was reduced, potentially due to the interaction of the EN1 iPep with EPRS. On the other hand, addition of exogenous proline substrate in excess re-established cell viability, suggesting that abrogation of protein synthesis caused mainly by the lack of proline led to amino acid starvation. A significant down-regulation of pro-inflammatory (CD69 and COL1A1) and up-regulation of anti-inflammatory (IL-11) as well as tumour suppressor (FOXA2) genes were observed in the EN1 active iPep treated cells. The above indicated that the EN1 iPep interfered with the prime function of EN1 and EPRS in SUM-149 cells, potentially by activating the amino acid response (AAR) pathway and regulating inflammatory genes. The EN1 iPep as a specific TF inhibitor may be an important therapeutic tool in treating EN1 expression related cancer and in addition other immuno-compromised diseases.

In chapter 5, I targeted another oncogenic TF, SOX2, which is known to be overexpressed in aggressive cancer cells. Cancer cell lines representing aggressive breast and ovarian cancers, namely the T11 mouse mammary carcinoma cell line, MDA-MB-435s breast cancer cells, and PA1 serous ovarian cancer (SOC) cells, which display high levels of SOX2 expression, were tested using a newly constructed SOX2 iPep possessing similar inhibitory properties to the EN1 iPep. In addition, breast cancer cell lines with lower SOX2 expression were examined. The SOX2 iPep showed high specificity dependent on the levels of SOX2 expression and the iPep dose, with strongest effect in T11 cells followed by PA1 and MDA-MB-435s, while the SOX2 iPep had no effect on the low-SOX2 expressing cancer cells and normal mammary cells such as HUMEC and MCF10A. Interestingly, the SOX2 iPep synergized with cisplatin by sensitizing T11 and PA1 cells to cisplatin treatment and achieving IC$_{50}$ values of 13.23 to 3.51 µM and 16.25 to 4.39 µM in T11 and PA1 cells, respectively. Both cisplatin and the SOX2 ipep induced differentiation of cancer cells by significantly
down-regulating SOX2 and other stem cell markers (NANOG, NESTIN, CD133, CD44, SSEA4 and CD49f) as well as reducing cell proliferation by 40-100%. At the same time, they induced up-regulation of differentiation markers (CD24, CK18 and β-tubulin) by 68-250%. This provided strong evidence that SOX2 targeting via iPep technology can have significant anti-cancer effects in aggressive breast and ovarian cancers.

The work of this thesis showed that cisplatin is a potent chemotherapeutic drug for TNBCs enriched in CSCs as it acts to push them towards a more differentiated phenotype. In order to reduce cisplatin dose, and thus its toxicity, but still maintain its potent anti-cancer effects, combination therapies are important. Towards this, we tested and were able to show a significant synergistic effect of cisplatin with one of the interference peptides tested that targets the SOX2 oncogene in breast and ovarian cancers enriched in CSCs. This cell population was targeted through the trickling of transcriptional and translational machinery using cisplatin and specific TF-targeting iPeps. These data highlight the importance of combinational therapies in successfully treating cancer, and provide strong evidence of the potential benefits of interference peptide technology in targeting specific tumour-seeding cells, without harming normal cells. This thesis sets the basis for future research to explore further, optimise and finally utilise this technology in combating cancer.
ACKNOWLEDGEMENTS

First and foremost, I thank GOD for giving me the strength and His blessings in order for me to successfully complete this thesis, which is an important part of my life.

Secondly I would like to express my heartfelt gratitude and sincere appreciation to my supervisors, Associate Professor Pilar Blancafort, Dr. Foteini Hassiotou and Professor Luis Filgueira for their guidance, assistance, patience and enthusiastic support throughout my candidature. I’m truly indebted to my supervisors for providing me the opportunity to work in the field of cancer research and sharing their knowledge which has helped to establish my research skills and will walk me through my future as a scientist.

I’m also pleased to acknowledge UWA for the facilities, Mr. Greg Cozens, Miss Celleste Wale, Mr. Guy Ben-Ary, Ms Vicki Wallis, Ms Alecia-Jane Twigger and Ms Jessie O’Mahony for their scientific and technical assistance.

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Praseetha Prabhakaran
1 March 2014
PUBLICATIONS

JOURNAL ARTICLES


CONFERENCES


SEMINAR

STATEMENT OF CANDIDATURE CONTRIBUTION

The majority of the work presented in this thesis was completed by the author Praseetha Prabhakaran. However, other individuals require acknowledgement for their contributions to each chapter and the publications arising from this thesis.

Chapter 2
This chapter is an overview/literature on the characteristics and promising treatments in triple negative breast cancers which discusses the current knowledge, briefly integrating some of the major findings of this thesis.

Chapter 3
I planned and designed the study, performed the measurements and data analyses, and wrote the manuscript for publication. Professor Filgueira and Dr. Hassiotou provided advice on the study design and critically reviewed the manuscript. Dr. Hassiotou contributed to the design, analysis and interpretation of the FACS experiments.

Chapter 4
I planned and designed the study, and performed the measurements and data analyses. Mr. Cozens assisted with the radioactive proline/methionine incorporation assays and measurements and contributed to Figure 4B of this chapter. A/Professor Blancafort designed and constructed the EN interference peptides (iPeps) (Figure 2A and 2C) and provided advice on the study design. Dr. Hassiotou contributed to interpretation of the data and critically reviewed the chapter.

Chapter 5
I planned and designed the study, and performed the measurements and data analyses. Ms Jessie O’Mahonny assisted with the SOX2 gene expression experiment and contributed Figure 2A of this chapter. A/Professor Blancafort designed and constructed the SOX2 interference peptide (iPep) (Figure 1A and 2B). A/Professor Blancafort and Dr. Hassiotou provided advice on the study design and interpretation of the data, and critically reviewed the chapter.

The candidate

The coordinating supervisor

Assoc. Prof. Pilar Blancafort
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<tr>
<td>AAR</td>
<td>Amino Acid Response Pathway</td>
</tr>
<tr>
<td>aaRS</td>
<td>Amino Acid tRNA synthetase</td>
</tr>
<tr>
<td>ABC</td>
<td>ATP Binding Cassette</td>
</tr>
<tr>
<td>AIMP</td>
<td>Multisynthesis Complex</td>
</tr>
<tr>
<td>BCSC</td>
<td>Breast Cancer Stem Cell</td>
</tr>
<tr>
<td>BRCA1</td>
<td>Breast Cancer Protein-1</td>
</tr>
<tr>
<td>BRCA2</td>
<td>Breast Cancer Protein-2</td>
</tr>
<tr>
<td>BrdU</td>
<td>5-bromo-2'-deoxyuridine</td>
</tr>
<tr>
<td>BRP1</td>
<td>Breast Cancer Resistance Protein-1</td>
</tr>
<tr>
<td>CSC</td>
<td>Cancer stem cells</td>
</tr>
<tr>
<td>COL1A1</td>
<td>Collagen Type 1 Alpha 1</td>
</tr>
<tr>
<td>DAPI</td>
<td>4,6-diamidino-2-phenyindole</td>
</tr>
<tr>
<td>DNA</td>
<td>Deoxyribonucleic Acid</td>
</tr>
<tr>
<td>ELISA</td>
<td>Enzyme-linked immunosorbent assay</td>
</tr>
<tr>
<td>EN1</td>
<td>Engrailed-1</td>
</tr>
<tr>
<td>EN2</td>
<td>Engrailed-2</td>
</tr>
<tr>
<td>ESC</td>
<td>Embryonic Stem Cell</td>
</tr>
<tr>
<td>EPRS</td>
<td>Glutamyl-prolyl-tRNA synthetase</td>
</tr>
<tr>
<td>ER</td>
<td>Estrogen</td>
</tr>
<tr>
<td>FACS</td>
<td>Flow Activated Cell Sorting</td>
</tr>
<tr>
<td>FBS</td>
<td>Fetal Bovine Serum</td>
</tr>
<tr>
<td>FDA</td>
<td>Food and Drug Administration</td>
</tr>
<tr>
<td>FITC</td>
<td>fluorescein isothiocyanate</td>
</tr>
<tr>
<td>FOXA2</td>
<td>Forkhead Box A2</td>
</tr>
<tr>
<td>GAIT Complex</td>
<td>IFN-γ-activated inhibitor of translation Complex</td>
</tr>
<tr>
<td>HER-2</td>
<td>Human Epidermal Growth Factor-2</td>
</tr>
<tr>
<td>HUMEC</td>
<td>Human mammary epithelial cell</td>
</tr>
<tr>
<td>hESC</td>
<td>Human embryonic stem cells</td>
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<tr>
<td>HRP</td>
<td>Horse Radish Peroxidase</td>
</tr>
<tr>
<td>IBC</td>
<td>Inflammatory Breast Cancer</td>
</tr>
<tr>
<td>IF</td>
<td>Immunofluorescence</td>
</tr>
<tr>
<td>IL</td>
<td>Interleukin</td>
</tr>
<tr>
<td>iPep</td>
<td>Interference peptide</td>
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<tr>
<td>MaSC</td>
<td>Mammary Stem Cell</td>
</tr>
<tr>
<td>MDR</td>
<td>Multi Drug Resistance</td>
</tr>
<tr>
<td>MFI</td>
<td>Mean Fluorescence Intensity</td>
</tr>
<tr>
<td>MTS</td>
<td>(3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium)</td>
</tr>
<tr>
<td>mRNA</td>
<td>Messenger Ribonucleic Acid</td>
</tr>
<tr>
<td>NMEs</td>
<td>New Molecular Entities</td>
</tr>
<tr>
<td>NNEA</td>
<td>Non-essential amino acids</td>
</tr>
<tr>
<td>OCT-4</td>
<td>Octamer Binding Transcription factor-4</td>
</tr>
<tr>
<td>PARP</td>
<td>Poly ADP Ribose Polymerase</td>
</tr>
<tr>
<td>PBS</td>
<td>Phosphate Buffered Saline</td>
</tr>
<tr>
<td>PFA</td>
<td>Paraformaldehyde</td>
</tr>
<tr>
<td>PR</td>
<td>Progesterone</td>
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<tr>
<td>qRT-PCR</td>
<td>(quantitative) Real-Time Polymerase Chain Reaction</td>
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<tr>
<td>Acronym</td>
<td>Description</td>
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<tr>
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<tr>
<td>SSEA4</td>
<td>Stage Specific Embryonic Antigen-4</td>
</tr>
<tr>
<td>SOC</td>
<td>Serous Ovarian Carcinoma(s)</td>
</tr>
<tr>
<td>SOX2</td>
<td>(Sex Determining Region Y)-Box 2</td>
</tr>
<tr>
<td>TF(s)</td>
<td>Transcription factor(s)</td>
</tr>
<tr>
<td>TNBC(s)</td>
<td>Triple Negative Breast Cancer(s)</td>
</tr>
<tr>
<td>TNF</td>
<td>Tumour Necrosis Factor</td>
</tr>
<tr>
<td>tRNA</td>
<td>Transfer Ribonucleic Acid</td>
</tr>
<tr>
<td>UWA</td>
<td>The University of Western Australia</td>
</tr>
<tr>
<td>WHO</td>
<td>World Health Organisation</td>
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CHAPTER 1

GENERAL INTRODUCTION
1.1 BACKGROUND

Breast cancer is one of the most common and widely affecting cancers among women and in some rare cases in men (Jemal, Siegel et al. 2006). An array of factors are used to determine if a woman is at high risk of breast cancer, including family history, race, age at first menstruation and number of children (Jemal, Siegel et al. 2006). Breast cancer is generally treatable if it is detected at a very early stage. To date, the Breast Cancer 1, early onset gene 1 \textit{BRCA1} (Miki, Swensen et al. 1994) and Breast Cancer 2, early onset (\textit{BRCA2}) (Wooster, Bignell et al. 1995) are two common breast cancer susceptibility genes that are well defined. The epidemiologic, phenotypic, and clinical profile of \textit{BRCA1}-associated breast cancers are well characterized and are distinct from sporadic breast tumours (Phipps, Li et al. 2010). The \textit{BRCA1} gene germline mutations are strongly associated with increased risks of basal-like breast cancer (Turner, Reis-Filho et al. 2007) and serous ovarian cancers (Thompson and Easton 2002). About 80-90% of breast cancers arising in the germline mutation comprise the basal-like subtype. In addition, microarray analyses have also shown prominent similarities in gene expression between \textit{BRCA1}-related cancers and sporadic basal-like cancers (Esteller, Silva et al. 2000; Turner, Tutt et al. 2004). Both basal-like breast cancer and serous ovarian cancer show down-regulation of \textit{BRCA1} gene expression through methylation of its promoter (Yehiely, Moyano et al. 2006; Bowtell 2010).

The diagnosis of breast cancer includes physical examination, a mammogram and histopathology of breast tissue biopsies. Women diagnosed with breast cancer face a variety of treatment options such as surgical resection, radiotherapy and chemotherapy. To date, 31 oncology New Molecular Entities (NMEs) received approval from the Food and Drug Administration (FDA) for the treatment of solid tumours. Of the 31, 23 drugs were approved for the treatment of common solid tumours, which include breast cancer (n=12), prostate cancer (n=4), lung cancer (n=2) and colorectal cancer (n=5), and are in clinical use (Richey, Lyons et al. 2009). Research efforts behind the development of new anti-cancer drugs are ongoing due to the low specificity, acquired resistance, toxicity and consequent side effects of the current treatments (Dagher, Johnson et al. 2004).

Anti-cancer drugs can have metal or non-metal base components. In recent years, metal-based compounds have demonstrated promising antitumor effects and have been used as modern cancer therapies either alone or in combination. Cisplatin is the first metal-based drug used for cancer treatment (Rosenberg, Vancamp et al. 1965;
Rosenberg, VanCamp et al. 1969). The molecular mechanism of action of cisplatin is the platinum-DNA adducts formation that leads to cytotoxicity of tumour cells. Cisplatin has been used quite extensively in treating various cancers, especially testicular, ovarian, cervical and also breast cancer (Basu and Krishnamurthy 2010; Pines, Kelstrup et al. 2011).

In breast cancer, cisplatin interestingly shows specific effects in treating the Triple Negative Breast Cancer (TNBC) subtype, which is commonly known to have one of the worst prognosis among the other breast cancer subtypes (Bosch, Eroles et al. 2010; Hastak, Alli et al. 2010; Tiwary, Yu et al. 2011). The basis of the specific susceptibility of TNBCs and the other invasive cancers to cisplatin is still unclear. However, the detection of stem cell-related gene overexpression in some cancers and the sensitivity of these genes towards cisplatin treatment gave rise to the idea that targeting cancer stem cells (CSCs) within the tumour could be a promising option for cancer therapy. The SOX2, OCT4 and NANOG transcription factors (TFs) are important key regulators of embryonic stem cell (ESC) identity, controlling both the self-renewal and pluripotential of ES cells (Ben-Porath, Thomson et al. 2008). Subpopulations of breast cancer cells have been shown to express ESC-associated genes as well as stem cell genes typical of normal mammary stem cells (MaSCs), thus to possess certain traits associated with normal stem cells (Andrews, Matin et al. 2005; Ben-Porath, Thomson et al. 2008; Karamboulas and Ailles 2013). The apparent parallel in gene expression between subpopulations of breast tumour cells with stem cell-like properties and normal mammary stem cells (MaSCs) has generated great interest in the possible link between them (Singh, Cook et al. 2010; Karamboulas and Ailles 2013). Some breast tumours, especially the TNBCs, and also ovarian tumours overexpress SOX2 (Karamboulas and Ailles 2013; Schwede, Spentzos et al. 2013). This aberrant SOX2 expression is associated with uncontrolled cell proliferation and progression of breast cancers (Karamboulas and Ailles 2013).

Based on this, we hypothesise that the presence of a cell subpopulation comprising cancer stem-like cells (CSCs) within breast tumours may be one of cisplatin’s important targets in relation to positive therapeutic responses. A similar improved response is seen in ovarian cancer patients treated with cisplatin (van Jaarsveld, Helleman et al. 2013). However, cisplatin is known to be very toxic and therefore it is often combined with other less toxic drugs, e.g. cisplatin and paclitaxel.
(Bogdanovic, Kojic et al. 2002; Yde, Gyrd-Hansen et al. 2007) in an effort to reduce toxicity of the treatment to normal cells, but maintain strong anti-cancer effects.

Some tumour cells, for example the TNBC and ovarian carcinoma cells become resistant to anti-cancer therapies after a few treatments which often leave the patients with less or no other treatment options. In addition to that, tumours enriched with CSCs are often more linked to acquired resistance as this subpopulation initiates a continuous cell renewal program which tend to protect the cells from harsh chemical intervention. In order to enhance the effectiveness of TNBC treatment, more targeted therapies need to be established to overcome the acquired resistance of these tumours to cisplatin and other cytotoxic-based therapies that are routinely used. Resistance could either be pre-existent (intrinsic resistance) or drug-induced (acquired resistance). Acquired resistance is most common with cisplatin treatment and it results in an unresponsive chemotherapy in patients (Florea and Busselberg 2011; Basu and Krishnamurthy 2010). Specific targeting and knock-down of oncogenes involved in maintaining the tumour-seeding CSCs would be an attractive option to selectively treat these tumours. Inhibition of SOX2 and its partners could help halt or limit the proliferative potential of CSCs and coax them towards a more differentiated, and thus more susceptible to chemotherapy, state reducing or ideally eliminating the chance for recurrence and/or metastasis.

1.2 RESEARCH OBJECTIVES

To give insight into the mechanisms through which cisplatin sensitises breast cancer cells to chemotherapy as a single agent and in combination with other agents, we examined the anti-cancer effects and mechanism of action of cisplatin and two different interfering peptides (iPeps) specifically designed to target the ENGRAILED-1 (EN1) or the (sex determining region-Y) box 2 (SOX2) nuclear TFs. We tested a variety of TNBC lines such as BT-549, SUM-149, MDA-MB-468, MDA-MB-435, MDA-MB-231 and T11, a p53 null mouse mammary carcinoma as well as PA1, a serous ovarian carcinoma cell line. Cisplatin has been used as the main chemotherapy agent and has shown specific sensitivity in treating TNBCs as well as ovarian cancers. The designing of iPeps has provided with a new novel method to target the “undruggable” via oncogenic TFs involved in the invasion, progression and metastases of cancer. We hypothesised that the combination therapy of these iPeps with cisplatin or even other drugs such as taxol could help increase the efficiency of chemotherapy.
The specific aims of this thesis were to:

1. Examine the mechanism of action of cisplatin in breast cancer cells (Chapter 3);
2. Investigate the effect of EN1 inhibition using EN1 iPep as a single agent and in combination with cisplatin in inflammatory breast cancer cells (Chapter 4);
3. Determine the effect of SOX2 inhibition using SOX2 iPep as a single agent and in combination with cisplatin in breast and ovarian cancer cells (Chapter 5).

I hypothesised that:

1. Cisplatin induces differentiation of breast cancer cells;
2. EN1 iPep interferes with the EN1 TF function by controlling the regulation of inflammatory proteins;
3. SOX2 iPep interferes with SOX2 TF function and induces differentiation of breast and ovarian cancer cells.
CHAPTER 2

LITERATURE REVIEW

Triple negative breast cancers:
Characteristics and promising treatments
2.1 Characteristics of Breast Cancer

Breast cancer is the most common cancer among women worldwide, amounting to 23% of the total cancer incidence (Jemal, Siegel et al. 2010). Most patients with familial breast cancer, similar to ovarian cancer, have germline mutations in the tumour suppressor genes Breast Cancer Type 1 (BRCA1) and Breast cancer Type 2 (BRCA2) (Welch and King 2001). These genes are involved in two essential cellular processes, mainly the DNA damage repair and transcriptional regulation (Welch and King 2001; Lehmann, Bauer et al. 2011). Recent studies have also shown that BRCA1 regulates the MaSC fate (Liu, Ginestier et al. 2008; Bai, Smith et al. 2013; Schwede, Spentzos et al. 2013). Much of the difficulty in treating breast cancer is due to the heterogeneity of breast tumours, which consist of a cellular hierarchy similar to the normal breast (Villadsen, Fridriksdottir et al. 2007; Visvader 2009; Hassiotou, Beltran et al. 2012; Hassiotou and Geddes 2012), including cancer cells with stem cell properties and more differentiated tumour cells (Prat, Parker et al. 2010). Breast cancer heterogeneity and cellular hierarchy has led to the identification of at least five molecular subtypes, which are distinguished based on their molecular and clinical characteristics, and their pathogenesis (Sotiriou and Pusztai 2009; Bosch, Eroles et al. 2010; Hastak, Alli et al. 2010; Al-Ejeh, Smart et al. 2011).

In recent years, the cancer genome sequencing and various other arrays have made it possible to reveal distinct breast cancer subgroups and their molecular drivers based on genomic analysis (Curtis, Shah et al. 2012). Genomic aberrations and variations have been identified in different breast cancer patients, leading to specific subtype identification. Banerji and co-workers showed various mutations across breast cancer subtypes which represented missense (n=3,153), silent (n=1,157), nonsense (n=242), splice site (n=97), deletions (n=194) and insertions (n=110) (Banerji, Cibulskis et al. 2012). The breast cancer subtypes are classified as claudin-low and basal-like, human epidermal growth factor receptor 2 (HER2) positive, luminal A, and luminal B tumours.

Breast cancer cell lines are further characterised based on their cellular morphology, tissue source, tumour classification and the presence or absence of estrogen receptor (ER), progesterone receptor (PR) and HER2 receptor expression. The BRCA1-associated breast tumours are frequently hormone receptor negative [68–90%
(ER−)], HER2 negative (Lakhani, Van De Vijver et al. 2002; El-Tamer, Russo et al. 2004; Palacios, Honrado et al. 2004; Banerji, Cibulskis et al. 2012; Curtis, Shah et al. 2012), they express mutant p53 (Lakhani, Van De Vijver et al. 2002; Palacios, Honrado et al. 2004), and have higher Ki-67 expression levels depicting greater cellular proliferation (Palacios, Honrado et al. 2004; Phipps, Li et al. 2010). As shown in Figure 1, breast cancer subtypes differ in their genomic complexity, genetic alterations and importantly, clinical prognosis (Banerji, Cibulskis et al. 2012, Gatza et al. 2010). These traits of breast cancer cells are used in the clinical setting to determine the most appropriate mode of treatment.

Figure 1: Characteristic expression patterns in the different breast cancer subtypes, including the Luminal, HER2, Basal, Immune, Cell adhesion, Mesenchymal/Extracellular matrix (ECM) and Proliferation gene clusters. Each coloured square represents the relative transcript abundance (in log2 space) with red=highest expression, black=average expression and green=lowest expression. Modified from (Prat and Perou 2011).

Luminal breast tumours (e.g. MCF-7 cells) are more differentiated and are often successfully treated with chemotherapy, indicating that more differentiated tumours are more susceptible to treatments (Powell 2012; Marjanovic, Weinberg et al. 2013). In contrast, the basal-like (e.g. MDA-MB-468 cells) and claudin-low subtypes (e.g. MDA-
MB-231 and BT-549 cells) are less differentiated, thus more difficult to treat, with poorer prognosis (Hastak, Alli et al. 2010; Holliday and Speirs 2011) (Figure 2) which can be linked to the lack of ER, PR and HER2 receptor expression (Hastak, Alli et al. 2010; Holliday and Speirs 2011; Tiwary, Yu et al. 2011; Byrski, Dent et al. 2012).

**Figure 2**: Breast cancer clinical-pathological characterization: Kaplan–Meier relapse-free survival and overall survival curves using the UNC337 data set. Claudin-low tumours have poor outcome compared to luminal A tumours, however there are no differences in survival between claudin-low and other subtypes with poor prognosis (basal-like, HER2 enriched and luminal B). Modified from (Prat and Perou 2011).

**Figure 3**: Epithelial mammary and breast cancer cellular hierarchy. (A) During normal development, symmetric stem cell self-renewal results in stem cell expansion. This process is tightly regulated by components of the stem cell niche. (B) Stem cells differentiate into bipotent progenitor cells that possess transitory amplifying capacity that undergoes further differentiation into myoepithelial and luminal restricted progenitor cells. (C) The restricted progenitor cells
finally differentiate and become matured myoepithelial and luminal cells. (D) Breast cancer subtypes can be categorized similar to the normal mammary hierarchy, with claudin-low at the top level known as CSCs, basal-like in the second level as cancer progenitor cells, Luminal A and B at the lowest level as the more differentiated cancer cells based on the heterogenous histological appearance of breast tumours (Modified from Blancafort, Juarez et al. 2011).

2.2 Stemness, Cancer and Breast Cancer Stem Cells

Most organs contain a small number of stem/progenitor cells that self-renew and give rise to the more differentiated functional cells in the organ (Clarke, Dick et al. 2006; Wong, Segal et al. 2008). The unique attributes of ESCs and adult tissue stem cells to self-renew and undergo multi-lineage differentiation into different cell types have formed the basic definitions of “stemness”. Upon cell division, a stem cell either undergoes asymmetric or symmetric division to produce one or two daughter cells with similar self-renewal capability to maintain or expand the stem cell population for long term clonal growth (Kreso and Dick, 2014).

2.2.1 Cancer and Cancer Stem Cells

Cancer is a complex disease initiated by cells possessing abnormalities, which undergo uncontrolled cell division and may become capable of invading other tissues. Evolution of cancer occurs through clonal expansion, genetic diversification and clonal selection within the acclimatised settings of tissue ecosystem (Greaves and Maley, 2012). Advanced genome sequencing facility has verified that cancer is a heterogeneous mixture of genetically discrete subclones that occur through branching evolution (Greaves and Maley., 2012; Burrell et al., 2013; Kreso and Dick., 2014). Apart from that, epigenetic modification (non-genetic) and tumour microenvironment are also important determinants that contribute to the functional heterogeneity and significant discrepancy in cellular function of a tumour (Nguyen et al., 2012; Hanahan and Coussens, 2012; Kreso and Dick, 2014). These determinants along with genetic diversities may influence stemness concurrently or independently over time by which different forces may impact the stemness properties of a cell which leads to tumour progression and chemoresistance (Kreso and Dick, 2014). The ability of cancers to grow indefinitely has directed the idea that cancer stemness and normal tissue stem cells may share some common underlying mechanisms. Although there are various modes of cancer therapies available now, failure in response to most of the treatments often
occurs after a few rounds of treatment due to recurrence of the disease and side effects (Karamboulas and Ailles 2013).

Increasing evidence shows that a small group of cancer cells possessing self-renewal capacity known as CSCs drive the tumour programming and are less sensitive to treatment, thus causing recurrence of the disease (Al-Hajj, Wicha et al. 2003; Singh, Clarke et al. 2004; Zhao, Bao et al. 2011). In the nineteenth century, Rudolph Virchow and Julius Conheim were the first researchers to affirm and enlighten the hypothesis of CSCs possessing similar properties to normal stem cells (Soltanian and Matin 2011). Primarily, CSCs were identified in haematological malignancies, and then in solid tumours including breast, ovarian, prostate, brain, liver, lung, and skin cancers (Zhao, Bao et al. 2011).

The CSC theory explains that this small population can arise from any cell within the tumour that acquires the ability to self-renew and aberrantly differentiate into defective cell types (Andrews, Matin et al. 2005; Soltanian and Matin 2011). Based on the CSC hypothesis, it can be further hypothesized that the persisting CSCs within tumours may be responsible for disease relapse and metastasis, giving rise to new tumours, particularly since they have been demonstrated to be resistant to treatments (Perou 2011; Sampieri and Fodde 2012). A possible explanation for this is that CSCs are slow cycling/quiescent and proliferate indefinitely (continuously self-renew) in order to maintain longevity by which consequently they may escape the chemotherapy that targets and destroys actively dividing cells (Maugeri-Sacca, Vigneri et al. 2011; Moore and Lyle 2011; Sampieri and Fodde 2012). This would leave CSCs unharmed and capable of re-initiating cancer development at a later time (Andrews, Matin et al. 2005). This is thought to be one of the main treatment obstacles faced by patients diagnosed with aggressive breast cancers.
2.2.2 Breast Cancer Stem Cells

Breast cancer stem cells (BCSCs) were discovered in 2003 by a group of scientists at the University of Michigan Comprehensive Cancer Centre, being the first to be identified in a solid tumour (Al-Hajj, Wicha et al. 2003). Hajj et al. reported that just a few CSCs were responsible for the growth initiation and spread of breast cancer. The breast tumour is likely to come back and spread malignant cells to other parts of the body if the cancer stem cells are not destroyed (Al-Hajj, Wicha et al. 2003). BCSCs comprise a cell subpopulation within the breast tumour that is responsible for the initiation, progression, chemotherapy resistance, and metastasis of the tumour (Soltanian and Matin 2011). BCSCs may originate from normal MaSCs, progenitor cells or even differentiated cells that acquire self-renewal ability become tumorigenic due to multiple genetic and epigenetic alterations (Wicha, Liu et al. 2006; Shafee, Smith et al. 2008). Possessing similar properties to normal MaSCs, BCSCs proliferate and undergo differentiation, resulting in the growth and heterogeneous histological appearance of breast tumours (Turashvili, Bouchal et al. 2007; Levina, Marrangoni et al. 2008). However, they have evidently lost those mechanisms that keep their division under control. Figure 3 shows the presence of a similar pattern in the normal mammary epithelial and breast cancer hierarchy, whereby the BCSCs share an ESC-like signature, which is often associated with the poorly differentiated, high-grade estrogen receptor (ER)-negative tumours (claudin-low and basal subtypes) that have the poorest clinical outcome (Perou 2011; Marjanovic, Weinberg et al. 2013). Such characteristics are often associated with the TNBC subtype.

2.2.3 Triple Negative Breast Cancer

TNBC is a breast cancer subtype that is known for its lack of expression of ER, PR and HER2 (Anders and Carey 2008; Keller, Lin et al. 2010; Prat, Parker et al. 2010; Lehmann, Bauer et al. 2011; Prat and Perou 2011). TNBCs affect approximately 15-20% of breast cancer patients (Ma, Ellis et al. 2013). TNBCs are very aggressive, undergo rapid progression and frequent visceral metastases (Anders and Carey 2008). Most of the BRCA1-associated breast cancers are TNBCs, comprising mostly basal-like and also claudin-low subtypes (Anders and Carey 2008; Prat, Parker et al. 2010; Prat and Perou 2011) as shown in Figure 4. TNBCs lack or have compromised BRCA1 gene
expression, which is important in the regulation of DNA double-strand break repair mechanisms (Welch and King 2001; Lehmann, Bauer et al. 2011; Bai, Smith et al. 2013), cell cycle checkpoint (Desmedt, Haibe-Kains et al. 2008) and also the mammary stem cell fate (Bai, Smith et al. 2013; Schwede, Spentzos et al. 2013).

Epidemiologic studies have shown high TNBC incidence among younger women <40 years of age, and also for those of the African ancestry (Anders and Carey 2008). It is usually difficult to treat, as there are no available targeted therapies and patients are solely dependent on chemotherapy which is cytotoxic (Carey, Winer et al. 2010; Perou 2011; Zhao, Bao et al. 2011; Fisher, Ma et al. 2012; Powell 2012; Marjanovic, Weinberg et al. 2013). These tumours are known to be fuelled by BCSCs (Keller, Lin et al. 2010; Lehmann, Bauer et al. 2011; Prat and Perou 2011) and are surprisingly sensitive to chemotherapy; however acquired resistance is established within a short treatment period which often results in low (5 years) overall survival rate (Bosch, Eroles et al. 2010; Hastak, Alli et al. 2010; Tiwary, Yu et al. 2011) This leaves no other treatment options for patients with TNBC. ESC-associated transcriptional regulators such as $\text{NANOG}$, $\text{SOX2}$ and $\text{OCT4}$ are often highly expressed in breast cancer subtypes enriched in BCSCs, while they are epigenetically silenced in the more differentiated subtypes (Soltanian and Matin 2011).
Figure 4: Triple-negative breast cancer intrinsic subtype: A clinical-pathological distribution (A) Distribution of intrinsic subtype within the triple-negative tumours with and without Claudin-low tumours. (B) Distribution of ER+/HER2+, ER−/HER2+, ER−/HER2− clinical groups in the Claudin-low and Basal-like subtypes. Modified from (Prat and Perou 2011).

2.2.4 Genes involved in Embryonic Stem Cell identity

In recent years, the role and involvement of TFs in cancers have come into light. TFs have important functions in the regulation of many genes that are associated with cancer initiation (oncogenes). \textit{SOX2}, \textit{OCT4} and \textit{NANOG} are the three main TFs, also known as master regulators of pluripotency, that are responsible for the maintenance of the undifferentiated state of ESC (Boyer, Lee et al. 2005; Yamanaka, Li et al. 2008; Leis, Eguiara et al. 2012). \textit{SOX2}, a member of the family of the High Mobility Group (HMG) domain (Lefebvre, Dumitriu et al. 2007), is a very important oncogene as it possesses similar key functions in \textit{SOX2}-expressing cancer cells as in normal stem cells, including control of cell self-renewal, regulation of cell development and multi-lineage differentiation. The \textit{SOX2} TF is believed to interact with its other primary partners, such as \textit{OCT4} of the Pit-Oct-Unc (POU) domain and \textit{NANOG}, and its many downstream
targets such as \textit{PAX6}, a process that when aberrantly regulated can lead to
tumorigenesis (Lefebvre, Dumitriu et al. 2007; Leis, Eguiara et al. 2012; Liu, Lin et al.
2013). \textit{SOX2} and \textit{OCT4} selectively interact with each other via the conserved POU and
HMG domains respectively, which then dimerize onto the DNA in specific
conformational arrangements (Remenyi, Lins et al. 2003). Aberrant \textit{SOX2} expression
has been observed in human cancers such as the small cell lung cancer, pancreatic
intrapertitoneal neoplasia, gastric, prostate, ovarian and basal-like breast cancer
is also overexpressed in \textit{BRCA1} germline mutated tumours (Rodriguez-Pinilla, Sarrio et
al. 2007). As \textit{BRCA1} is a tumour suppressor gene and has an important role in the
development of the normal mammary gland, it may also be involved in the regulation of
MaSC (Yehiely, Moyano et al. 2006). Hence, dysfunction in this gene possibly leads to
an early differentiation arrest while up-regulating expression of ESC genes. \textit{SOX2} has
been also implicated as a target in various signalling pathways during embryogenesis,
while its expression has been observed during the early stage of tumour formation and
is lost at differentiation in breast cancers (Leis, Eguiara et al. 2012). Recent studies have
shown that \textit{SOX2} and its primary partners regulate pluripotency as well as
differentiation not only through transcriptional, but also translational and post-
translational modifications (Liu, Lin et al. 2013). \textit{SOX2} and \textit{OCT4} selectively interact
with each other via the conserved POU and HMG domains respectively, which then
dimerize onto DNA in specific conformational arrangements (Remenyi, Lins et al.
2003).

Apart from its binding partners, the \textit{SOX2} TF also targets other downstream TFs.
The newly identified homeodomain (HD)-containing transcription factor (TF\textsubscript{HD}) known
as the \textit{EN1} is an important target of \textit{SOX2} and an oncogene (Beltran, Graves et al.
2013). The engrailed genes, \textit{EN1} and \textit{EN2}, are normally expressed in neural cells,
having a major role in the development of the central nervous system (Morgan, Boxall
et al. 2011; Beltran, Graves et al. 2013). Interestingly, \textit{EN1} and \textit{EN2} have recently been
highlighted as potential oncogenes in breast cancer (Mayor, Casadome et al. 2009;
Morgan, Boxall et al. 2011). \textit{EN2} is expressed in most breast cancers, however \textit{EN1} is
selectively overexpressed in basal-like breast cancers, which are often associated with
chemoresistance and poor clinical outcome (DiMeo, Anderson et al. 2009). Similarly in
colorectal cancer, hypermethylation of \textit{EN1} that was identified in stool and serum DNA
samples of colorectal cancer patients has been correlated with worse overall survival
The hypermethylation is an early event during tumorigenesis and disease progression (Mayor, Casadome et al. 2009). Many genes including tumour suppressor genes are silenced by promoter hypermethylation in tumours (Widschwendter and Jones 2002). The silencing of important genes plays important roles in carcinogenesis, by affecting the genome stability, cell-cycle entrance, proliferation and apoptosis (Esteller 2007; Mayor, Casadome et al. 2009). Previous studies have shown that the knock-down of EN1 in basal-like breast cancer cells results in selective apoptosis mediated by caspase-3 activation (Beltran, Graves et al. 2013). Recently, the bifunctional tRNA synthase, glutamyl prolyl-tRNA synthetase (EPRS), has been identified to bind to EN1 (Beltran, Graves et al. 2013) and this had suggested that EN1 could be involved in controlling transcript-specific protein synthesis. In immune cells, EPRS is part of the IFN-ɤ-activated inhibitor of translation (GAIT) complex and controls the synthesis of proteins involved in fibrosis, invasion, inflammation and angiogenesis, such as VEGFA, collagens and other targets. However, the role of EN1 and EN2 in controlling expression of inflammatory proteins by modulating EPRS activity has never been investigated before (Beltran, Graves et al. 2013).

Targeted inhibition of oncogenic TFs overexpressed in CSCs is an attractive option in the treatment of various cancers. Unfortunately, these TFs are of undruggable nature mainly because transcription is a nuclear event with restricted accessibility as well as possess versatile and well programmed functions. It is difficult to completely inhibit TFs and therefore there is an increasing need to develop a targeted therapy for these TFs.

2.2.4.1 Peptide Technology in Targeting Transcription Factors

Although TFs are conventionally considered as undruggable, agents have been developed that target various levels of transcriptional regulation, including DNA binding by transcription factors, protein–protein interactions, and epigenetic alterations (Yan and Higgins 2013). The use of peptides may be a promising option for specific targeted therapy especially peptides which are of small molecules that can be synthesized chemically at a large scale and can be made to possess high specificity (Zhang, Eden et al. 2012). Peptides in the past have been used mainly to increase the efficiency of drug delivery while reducing side effects in patients (Curnis, Gasparri et al. 2004). These peptides can be of various types possessing specific modes of action.
such as drug carriers, ligand-targeting and cancer-associated protease-targeting peptides (Zhang, Eden et al. 2012). In addition to these peptides, there are also interfering peptides which work in a competitive-inhibition manner. Therefore, disrupting the complex formation or dimerization of oncogenic TFs with binding partners is an effective strategy to influence transcription. Seo and co-workers showed that small interfering peptide (siPEP)-mediated strategy using *Arabidopsis* little zipper (ZPR) proteins was able to inhibit auxin response factor (ARF) TFs in crop plants (Seo, Hong et al. 2011). In 2013, the Blancafort laboratory was the first to demonstrate a novel EN1-targeted therapy through the development of iPep by specifically targeting and inhibiting the EN1 oncogene in basal-like breast cancer cells (Beltran, Graves et al. 2013). Peptide mimetics or small molecules that bind transcription factors or their co-regulators, which interrupts the protein-protein interaction, are potential therapeutic agents (Arora and Ansari 2009; Yan and Higgins 2013). A list of transcription targeting agents are listed in Table 1.

**TABLE 1:** Transcription targeting agents at different levels. (Modified from (Yan and Higgins 2013).

<table>
<thead>
<tr>
<th>Action</th>
<th>Targeting Agents</th>
<th>Examples</th>
</tr>
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<tbody>
<tr>
<td>Targeting binding of transcription factors to gene-specific promoters</td>
<td>• DNA-binding small molecules&lt;br&gt;• Polyamides&lt;br&gt;• Transcription factor (TF) decoys</td>
<td>• Mithramycin&lt;br&gt;• Antracyclines&lt;br&gt;• N-methylpyrrole (Py)&lt;br&gt;• N-methylimidazole (Im)&lt;br&gt;• Double strand or hairpin single strand oligodeoxynucleotides (ODNs)</td>
</tr>
<tr>
<td>Targeting protein–protein interactions involved in transcriptional regulation</td>
<td>• Peptide mimetics and stapling peptides&lt;br&gt;• Small molecules targeting protein–protein interactions</td>
<td>• S3-M2001 (STAT3 mediated transcription inhibitor)&lt;br&gt;• SAH-p53-8 (p53 activator)&lt;br&gt;• Myc-MAX&lt;br&gt;• P53-MDM2</td>
</tr>
<tr>
<td>Epigenetic interventions in transcription</td>
<td>• DNMT inhibitors&lt;br&gt;• HDAC inhibitors&lt;br&gt;• HMT inhibitors</td>
<td>• 5-azactidine &amp; Decitabine&lt;br&gt;• SAHA &amp; VPA&lt;br&gt;• BIX-01294 &amp; Dot1</td>
</tr>
<tr>
<td>Artificial transcription factors for gene-specific transcriptional regulation</td>
<td>• Zinc Finger Proteins (ZFP)</td>
<td>• MASPIN&lt;br&gt;• VEGFA</td>
</tr>
</tbody>
</table>

*DNMT-DNA methyltransferases; HDAC-Histone deacetylases; HMT-Histone methyltransferases; VEGFA-vascular epidermal growth factor-A*
Transcription therapy via peptide technology is an emerging strategy that anticipates rectifying abnormal gene expression in cancer cells through direct intervention in the transcription process. It is estimated that at least 10% of FDA-approved anti-cancer drugs actually regulate the targeted transcription process in one way or another (Ghosh and Papavassiliou 2005; Yan and Higgins 2013).

2.2.5 Breast Cancer Stem Cells and Inflammation
Apart from the involvement of TFs, most recent studies have also demonstrated that TNBCs interestingly have reliance upon inflammatory genes. Harman and co-workers identified 32 inflammation-related genes (cytokines and chemokines) which are differentially expressed in TNBCs, with 10 of these genes playing crucial roles in anchorage-independent growth, and 24 genes being highly expressed in ER(-)/basal-like breast cancer cell lines (Li, Gonzalez-Angulo et al. 2011; Hartman, Poage et al. 2013). Inhibition of the proinflammatory cytokines Interleukin (IL)-6 and (IL)-8 resulted in inhibition of tumour colony formation and cell survival (Hartman, Poage et al. 2013). Triple negative inflammatory breast cancer (TN IBC) is the most aggressive type of breast cancer, hence very challenging to treat as is unresponsive to endocrine and anti-HER-2 therapies (Li, Gonzalez-Angulo et al. 2011).

Recently, it has been shown that there is a link between inflammation and cancer development (Karkoya, Liu et al. 2011; Beltran, Graves et al. 2014). Parallel to normal stem cells, CSCs interact and are regulated in their environment (niche), processes that involve inflammatory cytokines and chemokines through bidirectional paracrine signalling which involves cell to cell communication (Iliopoulos, Fabbri et al. 2007; Liu, Lu et al. 2011; Korkaya, Liu et al. 2011b; Hartman, Poage et al. 2013). Activation of stem cell pathways such as the Notch, Hedgehog, Wnt, NF-κB and Jak/Stat3 in breast cancer stimulates production of cytokines and chemokines, which leads to BCSC proliferation (Korkaya, Liu et al. 2011b). Increased expression levels of cytokines such as interleukins (IL)-1, (IL)-6, (IL)-8 and chemokines (CXCL1/2) has also been shown to promote angiogenesis, tumour growth and metastasis, resulting in resistance to chemo/radiotherapy and poor survival (Korkaya, Liu et al. 2011a; Korkaya, Liu et al. 2011b; Hartman, Poage et al. 2013). These signalling pathways activated during tumour formation are similar to the normal wound healing process (Coussens and Werb 2002; Kalluri and Zeisberg 2006). In breast cancer, recurrence after primary therapy has been correlated with high levels of serum C-reactive protein/β-myeloid (CRP) chronic
inflammation (Pierce, Ballard-Barbash et al. 2009; Korkaya, Liu et al. 2011a; Korkaya, Liu et al. 2011b). An association between ulcerative colitis, hepatitis C and chronic pancreatitis and the development of colon, liver and pancreatic cancers (Korkaya, Liu et al. 2011a) as well as gastric and bowel cancers (Mantovani, Marchesi et al. 2008) have also been epidemiologically demonstrated. As interleukins and chemokines seem critical in the development of cancer through the interaction and regulation of CSCs, they could be used as tools in targeted therapies for breast cancer as well as other cancers, especially the ones enriched in CSCs.

2.3 Connection between Ovarian and Breast Cancers

Breast cancer is the most frequent cancer while ovarian cancer is the fifth most frequent cancer among women in the world (Jemal, Siegel et al. 2010) Similar to the basal-like and TNBCs, ovarian cancer is also initially sensitive to chemotherapy, but it relapses eventually becoming increasingly aggressive (Schwede, Spentzos et al. 2013). Basal-like, TNBC and serous ovarian carcinoma (SOC), a specific subtype of ovarian cancer have a biological connection. First, the ovary and the breast, both being reproductive organs, are estrogen-responsive (Pal, Permuth-Wey et al. 2005; Domingo, Guillen et al. 2012). Extensive studies have been done on the status of ER and PR hormone receptors in breast cancers. However, not much is known about these receptors and their link to tumorigenesis in ovarian cancer patients. The loss of ER and PR receptors in basal-like and TNBC and in high-grade serous carcinomas has been associated with low survival rate (5 years) and poor prognosis (Sieh, Kobel et al. 2013). Most familial basal-like breast cancers and ovarian cancers undergo germline mutations in **BRCA1** and **BRCA2** and are high-risk cancers (Welcsh and King 2001; Antoniou, Pharoah et al. 2003; Schwede, Spentzos et al. 2013). Often, breast and ovarian cancer patients with **BRCA1** and **BRCA2** mutations are also associated with somatic mutations of the **p53** tumour suppressor gene (Welch and King 2001). In addition to that, both TNBCs and ovarian tumours are enriched in stem-like cell subpopulations (Lord and Ashworth 2010; Maugeri-Sacca, Bartucci et al. 2012; Schwede, Spentzos et al. 2013). About 80% of ovarian cancer subtypes and 15% of breast cancer subtypes consist of a population of CSCs (Schwede, Spentzos et al. 2013). This may explain the difficulty in treating these cancers and their sensitivity to DNA-damaging drugs, for example the platinum-based drugs as well as PARP inhibitors that inhibits the DNA repair system (Welch and King 2001; Antoniou, Pharoah et al. 2003; Schwede, Spentzos et al. 2013). It is thus
crucial to identify a potent targeted therapy for both TNBC and ovarian cancers as these cancers are a major cause of death among women worldwide.

2.4 Platinum compound in Breast cancer treatment

2.4.1 Platinum-based drugs

Metal compounds have been used for treatment of various diseases for centuries, although the molecular mechanism of their activity has never been fully understood (Sneader 2005). Half a century ago (Milacic, Chen et al. 2006), it was observed that metal ions were capable of binding to nucleic acids, thereby altering their conformation and biological function (Milacic, Chen et al. 2006). Some metals, such as vanadium, iron and molybdenum, form part of the living organism and are needed as essential micronutrients for health and survival (Crans, Woll et al. 2013). However, other metals such as lead, cadmium and arsenic, are highly toxic (Llanos and Ronco 2009). Metals with high reactivity properties such as platinum have become important diagnostic or therapeutic agents (Heffeter, Jungwirth et al. 2008). The very first metal used as an anti-cancer drug began with the discovery of a platinum complex called cisplatin (cis-diamminedichloroplatinum(II), cis-Pt(NH\textsubscript{3})\textsubscript{2}Cl\textsubscript{2}) (Figure 5) in 1969 by Rosenberg and coworkers (Rosenberg, VanCamp et al. 1969). Cisplatin has arisen as an important anti-cancer drug due to its promising success in the treatment of various cancers that are not responsive to other drugs. Nevertheless, there are still major problems related to its side-effects such as nephrotoxicity and acquired resistance, despite its successful clinical use for the past four decades (Stordal and Davey 2007).

2.4.2 Cisplatin

The discovery of cisplatin is a key event in the history of drug discovery (Hargrave-Thomas, Yu et al. 2012) that has been used extensively in the past four decades for the treatment of many cancers (Nishiyama, Okazaki et al. 2003; Dickson, Carvajal et al. 2011) including breast, testicular, ovarian, cervical, head and neck, and small cell lung cancers (Basu and Krishnamurthy 2010; Florea and Busselberg 2011; Pines, Kelstrup et al. 2011). Particularly in breast cancer, cisplatin has been used in combination with other drugs, such as taxanes, vinca alkaloids, and 5-fluorouracil (Florea and Busselberg 2011; Holliday and Speirs 2011) resulting in synergistic or additive effects.
2.4.2.1 Cellular Mechanism of Cisplatin

The cellular mechanism of cisplatin action is an important field of interest for research as it has promising anti-cancer effects, however not much is known of how it works in different cancers. Cisplatin has been shown to cause apoptosis in breast and colon cancer and in osteosarcoma (Boyle, Ma et al. 2006). Previous studies and up to date research have demonstrated that cisplatin targets the cellular DNA. Cisplatin is known to cause DNA damage by forming Pt-DNA adducts (Wang and Lippard 2005; Wang, Milum et al. 2011). The platinum atom forms a divalent bond with the purine bases at N7, whereby the 1,2 or 1,3 intra-strand crosslinks take place, and disrupt the DNA structure (Wang and Lippard 2005). However, the intra-strand 1,2-d(GpG) crosslink is the most frequent kind of adduct formed and this crosslink between two adjacent G residues has been shown to be the main lesion causing cytotoxicity (Figure 6), leading to the activation of various signal transduction pathways (Zeidan, Jenkins et al. 2008; Basu and Krishnamurthy 2010; Florea and Busselberg 2011; Wang, Milum et al. 2011).

![Figure 5: Cisplatin chemical structure.](image)

![Figure 6: Known cisplatin mechanism of action. Modified from (Boulkas, Pantos et al. 2007).](image)
CHAPTER 2: LITERATURE REVIEW

Cisplatin has been reported to kill cells through signalling pathways that are regulated by mismatch repair and p53 activation independently to promote cell death (O'Brien and Brown 2006; Shiu, Chang et al. 2007; Basu and Krishnamurthy 2010). The formation of DNA adducts leads to replication arrest and cell cycle checkpoint activation followed by sustained G2 phase arrest (Koprinarova, Markovska et al. 2010). The cell cycle is crucial in cancer treatment as some drugs induce cell death or cell arrest by interrupting the cell cycle process (Mueller, Schittenhelm et al. 2006; Koprinarova, Markovska et al. 2010). If the damage is too severe, cells undergo programmed cell death or apoptosis (O'Brien and Brown 2006). In testicular germ cell tumours (GCTs), treatment with cisplatin is partially linked to apoptosis induction by a p53-mediated G1/S phase cell cycle arrest through the transactivation of p21, a gene involved in cell cycle arrest (Mueller, Schittenhelm et al. 2006; Rizzo, Evangelista et al. 2011). Expression of p21 inhibited retinoblastoma gene product phosphorylation which in turn blocked the entrance into the S phase (Mueller, Schittenhelm et al. 2006). It was also demonstrated in this paper that in unsynchronized GCTs cells and the breast cancer cell line MCF-7 treated with cisplatin for two hours, the cells were arrested at G2/M phase after 28 hours, with reversible cell cycle arrest at sublethal doses (up to 4.5 and 20 µM respectively for the two cell types). The cells were then able to re-enter the cell cycle. In contrast, at high cisplatin dose (50 µM), MCF-7 cells accumulated and remained in G2 arrest, whereas GCTs cells at the high dose of 10 µM progressed to apoptosis out of the G2/M arrest. Exposure of the GCTs cells in G2/M with 10 µM cisplatin resulted in a higher apoptotic index (70%) compared to cells in G1 (34%) after 70 hours (Mueller, Schittenhelm et al. 2006). From these data, it can be concluded that cisplatin disrupts mitosis at the G2/M phase.

The cisplatin-DNA adducts interfere with DNA replication as well as transcription but the exact mechanism of action and specificity are still not established (Bogdanovic, Kojic et al. 2002; O'Brien and Brown 2006; Basu and Krishnamurthy 2010). In addition to cisplatin forming DNA adducts, preliminary work in this thesis has demonstrated that this drug also has an effect on the cell cytoskeleton by disrupting the microtubule, similar to the drug taxol (Zeidan, Jenkins et al. 2008), with cisplatin causing marked changes in cell morphology and actin cytoskeleton in MCF-7 cells. A similar observation was also seen in MDA-MD-468 breast cancer cells (Figure 7). There were distinct morphological changes upon 20 µM cisplatin treatment such as increased cell
size and microtubule disruption. The microtubules had frozen-like appearance which could be due to tubulin polymerization, hence the increased expression of β-tubulin.

2.4.2.2 Transport of cisplatin into Tumour Cells
Cisplatin is thought to enter into cells through either passive diffusion, facilitated diffusion or active transport by multiple transporters, for example the Na\(^+\) and K\(^+\) channels which are ATPase carrier-mediated and the Ctrl which is a solute carrier transporter (Basu and Krishnamurthy, 2010). It is known that cisplatin acts on two main cell death pathways in cancer cells. The extrinsic pathway is receptor-mediated and it involves specific ligands binding to TNF-α receptor and resulting in activation of pro-caspase-8 (Wang and Lippard 2005; Shiu, Chang et al. 2007; Basu and Krishnamurthy 2010). Secondly, the intrinsic pathway, which is mitochondria-mediated, is activated by the release of cytochrome C due to mitochondrial disruption caused by cell stress (Shiu, Chang et al. 2007; Basu and Krishnamurthy 2010).

![Figure 7](image-url)

**Figure 7:** Cisplatin influences expression of cytoskeletal markers and cell morphology in MDA-MB-468 breast cancer cells. B-tubulin was upregulated upon 20 µM cisplatin in MDA-MB-468 breast cancer cells. (A) Control and (B) 20 µM cisplatin treated cells. Dapi nuclear stain was used to stain the nucleus (blue), phalloidin to stain actin (red) and β-tubulin to stain microtubule (green). Scale bars: 50 µm.

2.4.2.3 Cisplatin Analogues
Carboplatin and oxaliplatin, analogues of cisplatin (Figure 8), have also shown important chemotherapeutic characteristics whereby carboplatin is less toxic compared to cisplatin though it has similar DNA lesion formations (Heffeter, Jungwirth et al. 2008). Oxaliplatin on the other hand is commonly used to treat cisplatin-resistant breast...
tumours (Raymond, Faivre et al. 2002). However, some studies have shown that oxaliplatin works effectively only in certain situations such as in combination therapy with cisplatin rather than as a single drug, since it had very low positive effects in cisplatin-resistant malignancies (Giacchetti 2002; Stordal and Davey 2007). These analogues somehow cause cross-resistance with cisplatin, hence lead to lower sensitivity in tumour cell lines (Raymond, Faivre et al. 2002; Oliver, Mercer et al. 2010).

Many combination therapies have been carried out with cisplatin and other non-metal drugs, such as taxol and solamargine (steroid alkaloids) (Zeidan, Jenkins et al. 2008) and genisten and sodium butyrate (histone deacetylase inhibitor) (Jawaid, Crane et al. 2010; Koprinarova, Markovska et al. 2010), however it is rarely combined with other metals (Shiu, Chang et al. 2007). Most studies find that cisplatin works more effectively in a combination therapy with other drugs in breast cancer (Boyle, Ma et al. 2006; Shiu, Chang et al. 2007; Yde, Gyrd-Hansen et al. 2007; Jawaid, Crane et al. 2010), lung cancer (Shiu, Chang et al. 2007) and cervical cancer (Koprinarova, Markovska et al. 2010).

2.5 Conclusions
Efficient treatments for breast cancer patients are urgently needed, as this disease is one of the most deadly diseases in women. Breast cancer heterogeneity and cellular hierarchy has led to the specification of at least five molecular subtypes with differing severity and hence respective modes of treatment. Recent findings reveal the presence of CSCs in breast cancers, which are linked to increased cancer invasiveness. A similar occurrence of CSCs is observed in ovarian cancers. The most recently identified TNBC
subtype and SOCare highly enriched in CSCs and related to multiple deaths of women worldwide. Currently, the unavailability of targeted therapies for these cancers is a major setback in effectively helping these patients. As current treatments are largely ineffective, the development of novel effective therapeutics and/or the improvement of the current treatments are crucial in order to treat this disease and to halt cancer progression and metastasis.

To date, the platinum based anti-cancer drug cisplatin is the most effective chemotherapy agent for the treatment of TNBCs. The special sensitivity of TNBCs to cisplatin would be expected to increase the treatment response and relapse-free survival rates of patients. However, the increased acquired resistance incidence with cisplatin has led to combination therapy with other possible drugs to enhance the efficacy of treatments. As TNBCs are enriched in cells with cancer stem-like cell properties that are responsible for the resistance to treatment and recurrence and metastasis of the disease, selectively targeting this particular cell subpopulation within the breast tumour may be an important method of cancer treatment in these and other cancers with the same properties. It is a known fact that the ESC identity is controlled by a group of TFs such as the SOX2, OCT4 and NANOG and these genes could be among the key solutions offering unique opportunities for targeted therapies in TNBCs enriched in CSCs expressing one or more of these TFs.
CHAPTER 3

CISPLATIN INDUCES DIFFERENTIATION OF BREAST CANCER CELLS

CHAPTER 3: CISPLATIN DIFFERENTIATES BREAST CSCs

Cisplatin induces differentiation of breast cancer cells

3.1 Abstract
Breast tumours are heterogeneous including cells with stem cell properties and more differentiated cells. This heterogeneity is reflected into the molecular breast cancer subtypes. Breast cancer stem cells are resistant to chemotherapy, thus recent efforts are focusing on identifying treatments that shift them towards a more differentiated phenotype, making them more susceptible to chemotherapy. Potential of the drug cisplatin to induce differentiation was examined in breast cancer cell lines that represent different breast cancer subtypes. We used three cell lines representing triple-negative breast cancers, BT-549 and MDA-MB-231 (claudin-low) and MDA-MB-468 (basal-like), along with oestrogen and progesterone receptor positive MCF-7 cells (luminal). Cisplatin was applied at 2.5, 5, 10 and 20 µM, and cell viability and proliferation were measured using MTS and BrdU assays, respectively. The effect of cisplatin on the cellular hierarchy was examined by flow cytometry, immunofluorescence and qRT-PCR. Cisplatin treatment of 10 and 20 µM reduced cell viability by 36-51% and proliferation capacity by 36-67%. Treatment with cisplatin resulted in 12-67% down-regulation of stem cell markers (CD49f, SSEA4) and 10-130% up-regulation of differentiation markers (CK18, SMA, β-tubulin). At the mRNA level, CD49f was down-regulated whilst β-tubulin was up-regulated in the claudin-low cell lines. SSEA4 protein expression decreased upon cisplatin treatment, but SSEA4 mRNA expression increased indicating a differential regulation of cisplatin at the post-transcriptional level. Cisplatin reduces breast cancer cell survival and induces differentiation of stem/progenitor cell subpopulations within breast cancer cell lines. These effects indicate the potential of this drug to target specific chemotherapy-resistant cells within a tumour.

3.2 Introduction
Breast cancer is one of the most frequent cancers among women worldwide (Jemal, Siegel et al. 2010). Much of the difficulty in treating this disease is due to the heterogeneity of breast tumours, which consist of a cellular hierarchy similar to the normal breast (Villadsen, Fridriksdottir et al. 2007; Visvader 2009; Hassiotou, Beltran et al. 2012; Hassiotou and Geddes 2012), from cancer cells with stem cell properties to more differentiated tumour cells (Prat, Parker et al. 2010). BCSCs comprise a cell subpopulation within a tumour that is responsible for the initiation, progression,
CHAPTER 3: CISPLATIN DIFFERENTIATES BREAST CSCs

Chemotherapy resistance, and metastasis of the tumour (Clarke, Dick et al. 2006; Croker and Allan 2008; Short and Curiel 2009; Monteiro and Fodde 2010; Perou 2011; Zhao, Bao et al. 2011; Dave, Mittal et al. 2012; Sampieri and Fodde 2012). The BCSCs may have originated from either normal mammary stem cells (MaSCs), progenitor cell or more differentiated cells that acquire self-renewal ability and have become tumorigenic due to multiple genetic and epigenetic changes, however the exact origin is still unclear (Wicha, Liu et al. 2006; Shafee, Smith et al. 2008). Possessing similar properties to normal MaSCs, BCSCs proliferate and undergo multi-lineage differentiation, resulting in the growth and heterogeneous histological appearance of breast tumours (Turashvili, Bouchal et al. 2007; Levina, Marrangoni et al. 2008).

Breast cancer heterogeneity and cellular hierarchy has led to the identification of five molecular subtypes, which are distinguished based on their molecular and clinical characteristics, and pathogenesis (Sotiriou and Pusztai 2009; Bosch, Eroles et al. 2010; Hastak, Alli et al. 2010; Al-Ejeh, Smart et al. 2011). These include the poorly characterised claudin-low tumours, the basal-like, HER2 positive, luminal A, and luminal B tumours.

Luminal breast tumours, represented in vitro by MCF-7 cells, are more differentiated and are often successfully treated with chemotherapy, indicating that more differentiated tumours are more susceptible to treatments. In contrast the basal-like (e.g MDA-MB-468 cells) and claudin-low subtypes (e.g. MDA-MB-231 cells) are less differentiated, difficult to treat with poor prognosis (Hastak, Alli et al. 2010; Holliday and Speirs 2011). Often, basal-like and claudin-low tumours lack the ER, PR and HER2 receptors, and are thus triple negative (Hastak, Alli et al. 2010; Holliday and Speirs 2011; Tiwary, Yu et al. 2011; Byrski, Dent et al. 2012). These tumours are fuelled by BCSCs, are highly resistant to chemotherapy (Hastak, Alli et al. 2010; Tiwary, Yu et al. 2011), very proliferative with worst survival rates (Bosch, Eroles et al. 2010; Hastak, Alli et al. 2010; Tiwary, Yu et al. 2011). Thus, recent efforts have been focusing on treatments that may shift the less differentiated BCSCs towards a more differentiated phenotype, making them more susceptible to treatment options, and eliminating the chance for recurrence and/or metastasis.

Cisplatin (cis-diamminedichloroplatinum II) is a metal-based anti-cancer drug (Rosenberg, Vancamp et al. 1965; Rosenberg, VanCamp et al. 1969) that has been used extensively in the past four decades for the treatment of many cancers (Nishiyama, Okazaki et al. 2003; Dickson, Carvajal et al. 2011), including breast, testicular, ovarian,
cervical, head and neck, and small cell lung cancers (Basu and Krishnamurthy 2010; Florea and Busselberg 2011; Pines, Kelstrup et al. 2011). Particularly in breast cancer, cisplatin has been used in combination with other drugs, such as taxanes, vinca alkaloids, and 5-fluorouracil (Florea and Busselberg 2011; Holliday and Speirs 2011), resulting in synergistic or additive effects. Cisplatin is known to cause DNA damage by forming Pt-DNA adducts at the 1,2-intrastrand crosslink, leading to the activation of various signal transduction pathways (Zeidan, Jenkins et al. 2008; Basu and Krishnamurthy 2010; Florea and Busselberg 2011; Wang, Milum et al. 2011). However, its exact mechanism of action and specificity are still not well established. To give insight into the mechanisms through which cisplatin sensitises breast cancer cells to chemotherapy, we examined the effects of cisplatin on cell phenotype and survival using four human cancer cell lines representing different molecular and differentiation subtypes of breast cancer.

3.3 Materials and Methods

3.3.1 Cell Culture

BT-549, MDA-MB-231, MDA-MB-468 and MCF-7 cells (American Type Culture Collection, ATCC) (Table 1) were cultured in T25 flasks (Corning, Tewksbury MA, USA) at 37°C and 5% CO₂ in DMEM/F12+glutamax™1 (Invitrogen, Carlsbad, CA, USA), supplemented with 20% fetal bovine serum (FBS) (Serana, WA Pty Ltd, Bunbury, WA, Australia), and 1% antibiotic-antimycotic (100 U/mL penicillin, 100 µg streptomycin/0.25 µg/mL) (Invitrogen). BT-549 and MDA-MB-231 cells were passaged twice a week, whilst MDA-MB-468 and MCF-7 once a week at 60-70% cell confluency.

<table>
<thead>
<tr>
<th>Cell line</th>
<th>Tumour type</th>
<th>Tumour classification</th>
<th>State of differentiation</th>
</tr>
</thead>
<tbody>
<tr>
<td>BT-549</td>
<td>Papillary invasive ductal carcinoma</td>
<td>Claudin-low</td>
<td>Less differentiated</td>
</tr>
<tr>
<td>MDA-MB-231</td>
<td>Adenocarcinoma</td>
<td>Claudin-low</td>
<td>Least differentiated</td>
</tr>
<tr>
<td>MDA-MB-468</td>
<td>Adenocarcinoma</td>
<td>Basal-like</td>
<td>Differentiated</td>
</tr>
<tr>
<td>MCF-7</td>
<td>Adenocarcinoma</td>
<td>Luminal A</td>
<td>Most differentiated</td>
</tr>
</tbody>
</table>
3.3.2 Determination of Cell Viability

Cell cultures of 60-70% confluency were used for experiments. $3 \times 10^3$ cells per 100-µL and well were seeded in flat bottom 96-well plates (Sarstedt, Newton, USA). Cells were counted using a haemocytometer. After 24 hours, cisplatin was added at different concentration (2.5, 5, 10 and 20 µM) (Sigma-Aldrich, St Louis, MO, USA). These concentrations were chosen according to the range of cisplatin concentrations used in treatments (Persons, Yazlovitskaya et al. 1999; Zhang, Gao et al. 2003; Moore and Lyle 2011; McAuliffe, Morgan et al. 2012). The stock solution of cisplatin was prepared in 1 mg/mL Dimethylsulfoxide (DMSO) (Sigma-Aldrich) and further diluted with culture medium accordingly. After incubation for 24 hours, cell viability was assessed by (3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium) (MTS) colorimetric assay, using Cell Titer 96® Aqueous (Promega, Madison, USA), according to the manufacturer’s instructions. The MTS substrate was added (10-µL:100-µL medium) and incubated for 4 hours. Lastly, the MTS activity was read at 490 nm using a plate reader (Labsystems Multiskan RC). The cell proliferation assay was measured using the BrdU colorimetric assay (Roche Diagnostics, Mannheim, Germany), according to the manufacturer’s instructions. After cisplatin treatment, the supernatant was removed and cells were fixed with 100-µL fixdent for 30 minutes, followed by washing (3X) with 200-µL buffer solution. Next, cells were further incubated with 100-µL primary antibody for 4 hours followed by washing (3X) with 200-µL buffer solution and finally incubated with 100-µL secondary antibody for 30 minutes. Proliferating cells are stained blue. The BrdU activity was read at 405nm using the same plate reader. Experiments were done in quadruplicate in three independent experiments.

3.3.3 Immunofluorescence Microscopy

Cells ($3 \times 10^4$ cells per mL) were grown on coverslips in 24-well plates (Sarstedt) for 24 hours. 20 µM cisplatin was added and cells were incubated for 24 hours. Cells were then fixed with 1% paraformaldehyde (PFA) in PBS/2% sucrose, permeabilised with 0.1% Triton X-100 in PBS for 30 minutes, incubated overnight with primary antibodies (Suppl. Table 1), and then incubated for 4 hours with secondary antibodies (Suppl. Table 1) and 40, 6-diamidino-2-phenylindole (DAPI) (Roche, 1:100) for nuclear staining. Appropriate negative controls (secondary antibody only) were used.
imaged using an Olympus 1X71 inverted optical microscope and an upright Nikon Eclipse 90i microscope.

### 3.3.4 Protein Immunodetection by Flow Cytometry

Breast cancer cell cultures of 60-70% confluency were passaged by trypsinisation. A day later, the cells were treated with 20 µM cisplatin and incubated further for 24 hours. Adherent cells were then gently scraped and centrifuged at 1200 rpm for 5 minutes. Cells were fixed in 1% PFA in PBS/2% sucrose for 20 minutes at room temperature, and incubated with primary antibodies (Suppl. Table 1) for 1 hour at 4°C, followed by incubation with secondary antibodies (Suppl. Table 1) for 30 minutes at 4°C. All intracellular marker antibodies were prepared in permeabilisation solution (0.05% Tween-20 in PBS), whilst surface marker antibodies were prepared in 7% FBS in PBS. Appropriate negative controls (secondary antibody only) were also used. Data acquisition was done with a FACS Calibur Flow Cytometer (Becton Dickinson, New Jersey, USA), and 10,000 events were collected and analysed per sample. FlowJo was used for data analysis. Expression levels were analysed as the standardised difference in the Mean Fluorescence Intensity (MFI) between the control and the test. A Mean Fluorescence Intensity (MFI) threshold was formed to distinguish the levels of protein expression: MFI≤20=very low; 21≤MFI≤40=low; 41≤MFI≤60=medium; 61≤MFI≤80=high; 81≤MFI≤100=very high; and MFI≥101=extremely high.

### 3.3.5 mRNA Quantification by qRT-PCR

Breast cancer cell cultures of 60-70% confluency were passaged by trypsinisation. A day later, the cells were treated with 20 µM cisplatin and incubated further for 24 hours. Adherent cells were gently scraped and centrifuged at 12000 rpm for 5 minutes. Total cellular RNA was extracted from the cell pellet, homogenised using 1 mL RNAzol®RT (Molecular Research Center, Inc) and transferred to smaller ependorf tube (2.5 µL). Next, the DNA/protein is precipitated by addition of 400 µL nanopure water and incubated for 15 minutes on ice and followed by spinning at 12000 rpm for a further 15 minutes (4°C). The colorless solution on the upper layer containing RNA is then slowly removed and transferred to a new tube. Finally, the mRNA is isolated by adding 400 µL 75% ethanol, incubated for 10 minutes on ice and followed by spinning at 1200 rpm for 8 minutes (4°C). Lastly, the mRNA washes are done (3X) using 400 µL 75% ethanol at
8000 rpm for 3 minutes each (4°C) and dissolved in RNase free water. RNA quantity and quality were assessed with NanoDrop 1000 (NanoDrop, Wilington, DE). For each sample, 1 µg RNA was treated with RQ1 RNase-Free DNase (Promega, Madison, USA) and was reverse transcribed using MMLV (Promega) by incubating at 25°C (10 minutes), 55°C (50 minutes) and 70°C (15 minutes) using PTC-100™ Programmable Thermal Controller (MJ Research Inc.). The RT Reaction Clean-Up MoBio kit (MoBio Lab Inc., CA, USA) was used for cDNA clean up. The Brilliant SYBR green qRT-PCR Master Mix consisting of 5-µL of IQ™ SYBR® Green Supermix (BIO-RAD, CA. USA), 1-µL of each forward and reverse primers for each gene (Table 2), 1-µL of H$_2$O and 2-µL of cDNA were used to detect the relative abundance of transcripts. The conditions for all qRT-PCR reactions were as follows: 10 seconds at 95°C followed by 30 seconds at 54, 55 and 60 °C (Table 2), and 15 seconds at 72°C for 40 cycles. Validation was done by sequencing of the PCR products, analysis of the melting curves and use of β-actin as the positive control and non-template sample as the negative control.

**TABLE 2.** Primers used for RT-PCR (AT: annealing temperature).

<table>
<thead>
<tr>
<th>Gene</th>
<th>Forward primer</th>
<th>Reverse primer</th>
<th>AT (°C)</th>
</tr>
</thead>
<tbody>
<tr>
<td>SSEA4</td>
<td>5’ TGG ACG GGC ACA ACT TCA TC 3’</td>
<td>5’ GGG CAG GTT CTT GGC ACT CT 3’</td>
<td>54</td>
</tr>
<tr>
<td>CD49f</td>
<td>5’ ATG GAG GAA ACC CTG TGG CT 3’</td>
<td>5’ ACG AGA GCT TGG CTC TTG GA 3’</td>
<td>60</td>
</tr>
<tr>
<td>β-Tubulin 6</td>
<td>5’ AGG CTA CGT GGG AGA CTC G 3’</td>
<td>5’ GCC CTG GGC ACA TAT TTC T 3’</td>
<td>60</td>
</tr>
<tr>
<td>β-Actin</td>
<td>5’ CGG CAT TCA CGA AAC 3’</td>
<td>5’ GGG CAG TGA TCT CTT 3’</td>
<td>55</td>
</tr>
</tbody>
</table>

### 3.3.6 Statistical Analysis

Statistical analysis and graphical exploration of the data were done in Microsoft Excel. The Student’s paired t-test with a two-tailed distribution was used to compare cisplatin-treated and untreated breast cancer cells. The results are presented as mean±SD (MTS and BrdU) and mean±SEM (flow cytometry and qRT-PCR), as indicated in the corresponding figure legends. The significance is shown as follows: *p≤0.05; **p≤0.0005; ***p<0.0001.
3.4 Results

3.4.1 Cisplatin reduces viability and proliferation of breast cancer cells

Cisplatin showed dose-dependent effects in all tested cell lines. At lower doses (2.5-5 µM), cisplatin enhanced cell viability and proliferation in BT-549 and MDA-MB-231 cells, whilst a small decline in viability and proliferation was observed in MDA-MB-468 and MCF-7 cells. At the doses most commonly reached in tissues in clinical treatments (10-20 µM), significant reductions in cell viability and proliferation were observed in all cell lines, but at different rates, ranging from 36-51% for cell viability and 36-67% for proliferation (Fig. 1A-D). MCF-7 cells, which are the most differentiated cells used, showed most dramatic reductions in cell viability at 10 and 20 µM cisplatin (49% and 58%, respectively; \( p < 0.0001 \)), followed by MDA-MB-468 (48% and 51%, respectively; \( p < 0.0001 \)), MDA-MB-231 (43% and 45%, respectively; \( p < 0.0001 \)) and BT-549 (36% and 44%, respectively; \( p < 0.0001 \)). Similarly, 5 µM caused significant reductions in cell viability and proliferation, but only in the more differentiated MDA-MB-468 (9% and 24%, respectively; \( p < 0.001 \)) and MCF-7 (9% and 29%, respectively; \( p < 0.01 \)).

Cell proliferative capacity decreased more than cell viability in the cell lines at 10 and 20 µM: in MCF-7 60% and 74%, respectively (\( p < 0.0001 \)), in MDA-MB-468 58% and 66%, respectively (\( p < 0.0001 \)) and in MDA-MB-231 51% and 61%, respectively (\( p < 0.0001 \)). Exception to this was BT-549 cells, which showed reduction in proliferation that was similar to the reduction of viability (36% and 45%, respectively; \( p < 0.0001 \)), suggesting that the surviving cells may undergo differentiation. Upon cisplatin treatment, the less differentiated cells displayed a more differentiated phenotype through the expression of differentiation markers (Fig. 1B); BT-549 cells appeared more contracted and elongated (Fig. A), and MDA-MB-231 cells appeared slightly enlarged and elongated (Fig. 1B).

Morphological changes were not as prominent in the more differentiated MDA-MB-468 and MCF-7 cells. These results demonstrate that cisplatin treatment caused a reduction in both cell viability and proliferation by interfering in cellular functions through unknown mechanisms, with the most prominent effects at the cisplatin concentrations that are expected in the clinical setting.
3.4.2 Cisplatin induces differentiation of breast cancer cells

The effect of cisplatin on expression of a variety of key markers, such as SSEA4, CD49f, nestin, SMA, CK18 and β-tubulin, was examined using flow cytometry. A cellular hierarchy was observed in the untreated cell lines (Fig. 2A-D). Untreated BT-549, MDA-MB-231, MDA-MB-468 and MCF-7 cells expressed stem cell markers (SSEA4, CD49f, nestin), a myoepithelial marker (SMA), an epithelial marker (CK18) and a microtubule marker (β-tubulin). These markers were expressed at various levels depending on the differentiation status of each cell line (Fig. 2; Table 4). BT-549 expressed medium to high levels of β-tubulin, CD49f and nestin, but very low levels of SMA and CK18 (Fig.2, Suppl. Table 2). MDA-MB-231 cells expressed medium to high levels of CK18, β-tubulin, CD49f and SSEA4, whilst SMA and nestin were expressed at very low levels. In contrast, MDA-MB-468 cells and MCF-7 expressed CK18 at very high levels, whilst SSEA4, CD49f, and SMA were expressed at low levels (Suppl.Table 2). Nestin was highly expressed in MDA-MB-468, but at low levels in MCF-7. β-tubulin was expressed at medium and high levels, respectively, in these cell lines (Table 4).
Figure 1. Cisplatin reduces cell viability and proliferation in breast cancer cells. (A) BT-549, (B) MDA-MB-231, (C) MDA-MD-468 and (D) MCF-7 breast cancer cells cultured without or with 20 µM cisplatin for 24 hours. The bar charts show the effect of cisplatin at increasing concentrations (2.5, 5, 10 and 20 µM) on cell viability and proliferation in the BT-
CHAPTER 3: CISPLATIN DIFFERENTIATES BREAST CSCs

549, MDA-MB-231, MDA-MD-468 and MCF-7 respectively, which were determined by MTS and BrdU assays. Cells were seeded at $3 \times 10^3$ density and incubated with cisplatin for 24 hours. Experiments were done in quadruplicate in 3 independent experiments. Bars are presented as mean±SD (n=3). *$p \leq 0.05$; **$p \leq 0.0005$; ***$p < 0.0001$.

Treatment with cisplatin shifted the cellular hierarchy of these cell lines, causing distinct changes in cell phenotype and protein expression (Fig. 2; Table 4). The stem cell markers SSEA4 and CD49f were significantly down-regulated in all the TNBC cell lines, BT-549 ($p=0.023$ and $p=0.034$, respectively), MDA-MB-231 ($p=0.018$ and $p=0.036$, respectively) and MDA-MB-468 ($p=0.03$ and $p=0.018$, respectively) (Fig. 2A-C; Suppl. Table 2). A significant reduction in CD49f expression was also observed in MCF-7 ($p=0.025$), but a very small or negligible change in expression of SSEA4 (Fig. 2D; Suppl. Table 2). In addition to down-regulation of stem cell markers, cisplatin induced significant up-regulation of differentiation markers. β-tubulin was up-regulated in MDA-MB-231 ($p=0.044$), MDA-MB-468 (β-tubulin: $p=0.020$) and MCF-7 (β-tubulin: $p=0.034$), whilst CK18 expression increased significantly in MDA-MB-231 ($p=0.045$), and only marginally in the latter two cell lines (Fig. 2B-D; Table 4). In BT-549 cells, SMA ($p=0.014$) and β-tubulin ($p=0.020$) were up-regulated (Fig. 2A; Suppl. Table 2). Immunofluorescence imaging confirmed the increased expression of SMA, CK18 and β-tubulin upon cisplatin treatment (Fig. 3). These results provided evidence of differentiation induction in the examined breast cancer cells upon cisplatin treatment.

Figure 2. Cisplatin shifts breast cancer cells towards a more differentiated phenotype. Flow cytometric quantification of CD49f, SSEA4, nestin, SMA, CK18 and β-tubulin protein expression showed various levels in 4 different breast cancer cell lines. Panel A, B, C and D show flow cytometry histograms of expression of the above markers in BT-549, MDA-MB-231, MDA-MB-468 and MCF-7 (black dashed line: unstained control; grey line: FITC-stained untreated breast cancer cells; black line: FITC-stained 20 µM cisplatin-treated breast cancer cells). The bar charts show quantification of the level of expression based on the mean fluorescence intensity (MFI) standardised difference of cisplatin-treated (20 µM cisplatin for 24 hours) and untreated BT-549, MDA-MB-231, MDA-MD-468, and MCF-7 breast cancer cells. Bars are presented as mean fluorescence intensity (MFI) standardised difference±SEM (n=3). *$p \leq 0.05$. 


CHAPTER 3: CISPLATIN DIFFERENTIATES BREAST CSCs

A

BT-549

B

MDA-MB-231

C

MDA-MB-468

D

MCF-7

- Negative control
- Untreated
- 20 μM cisplatin

Mean Fluorescence Intensity

CD49f
SSEA4
Nestin
SMA
CK18
β-Tubulin

CD49f
SSEA4
Nestin
SMA
CK18
β-Tubulin

CD49f
SSEA4
Nestin
SMA
CK18
β-Tubulin

CD49f
SSEA4
Nestin
SMA
CK18
β-Tubulin

Mean Fluorescence Intensity
Figure 3. Cisplatin influences expression of differentiation markers in BT-549 and MDA-MB-468. Differentiation markers CK18 and β-tubulin were up-regulated upon treatment with 20 µM cisplatin in both BT-549 and MDA-MB-468 cells. The mouse anti-β-tubulin was used to stain microtubules (green), whilst DAPI nuclear stain was used to stain the nucleus (blue). The images were taken using Nikon microscope at 20X magnification. Scale bars: 100 µm.
3.4.3 Differential gene regulation by cisplatin at the transcriptional and post-transcriptional levels

To examine the role of cisplatin in gene regulation, CD49f, SSEA4 and β-tubulin mRNA expression levels were measured in 20 µM cisplatin-treated and untreated cells in two of the most invasive breast cancer cell lines, BT-549 and MDA-MB-231, which showed either low or very high protein expression levels prior to treatment, and were clearly differentiated by cisplatin. The mRNA expression of β-tubulin was significantly increased upon cisplatin treatment in both BT-549 ($p=0.043$) and MDA-MB-231 ($p=0.021$) (Fig. 4). mRNA expression of CD49f significantly decreased ($p=0.036$ and $p=0.034$, respectively) with cisplatin treatment (Fig. 4) while at lower cisplatin dose (5 µM), an increase (MDA-MB-231: $p=0.014$) in the mRNA expression was observed (Fig. 5) in both these cell lines. The mRNA expression pattern for both β-tubulin and CD49f were consistent with the protein expression pattern at 20 µM cisplatin treatment. However, SSEA4 mRNA expression was significantly up-regulated in cisplatin-treated BT-549 ($p=0.019$) and MDA-MB-231 cells ($p=0.022$) (Fig. 4), which was contrary to the protein expression pattern. This suggests that cisplatin may differentially regulate gene expression, with potential post-transcriptional effects for certain genes.

Figure 4. Effects of cisplatin on mRNA expression of β-tubulin, CD49f and SSEA4 in breast cancer cells. Individual PCR reactions were normalised against internal positive control (β-actin) and plotted as the mRNA relative expression for both untreated and cisplatin-treated BT-549 (A) and MDA-MB-231 (B) breast cancer cells. Cells were incubated with 20 µM cisplatin for 24 hours. Experiments were done in duplicates in 3 independent experiments. Bars are presented as mean±SEM. *$p\leq0.05$. 
3.5 Discussion
Recent efforts are focusing on the development of breast cancer treatments that specifically target the CSCs, which are responsible for tumour progression, metastasis and recurrence. The only drugs that have recently shown to specifically target CSCs are salinomycin (Naujokat and Steinhart 2012) and metformin (Rattan, Ali Fehmi et al. 2012). Given the resistance of CSCs to chemotherapy, successful treatments must first induce CSC differentiation to make them more susceptible to the killing effects of anti-cancer drugs. Here, we demonstrate cisplatin-induced differentiation of common cell lines representing different subtypes of breast cancer, including TNBCs, which are the most aggressive and highly populated by CSCs. This effect of cisplatin was possibly delivered via down-regulation of the stem cell markers CD49f and SSEA4 either at the mRNA or protein level, and subsequent cisplatin cytotoxicity resulting in marked reduction in cell viability and proliferation. These findings may give insight into the cellular hierarchy of breast tumours and suggest a novel mechanism of action for cisplatin that first differentiates and then kills breast cancer cells.
Cell viability assays of cisplatin treatment revealed a cytotoxic effect at high doses (10 and 20 µM). The observed increase in cell proliferation at low cisplatin doses suggests that the CSCs, which proliferative indefinitely within a tumour, are not killed, and this needs to be taken into consideration in future cisplatin treatments. At high doses, cisplatin probably kills the tumour cells by interfering with cellular structure and function at the DNA level (Basu and Krishnamurthy 2010), as has been shown previously. At the same time, cisplatin appeared to regulate gene expression, and therefore interfere with the stage of cellular differentiation, both at the mRNA and protein levels. Interestingly, the response to cisplatin treatment varied between the different cell lines tested, and for the different cisplatin doses examined. This is in agreement with previous studies showing that some tumour cells require high cisplatin doses, whilst other tumour cells require small doses to be killed (Milrot, Jackman et al. 2012; Foroodi, Duivenvoorden et al. 2009; Wang, Milum et al. 2011). Low, non-toxic dosage may have a different effect without causing cell death. This differential response suggests that cisplatin may act in a tumour-specific manner, depending on the properties and cellular hierarchy manifested by each tumour. This led us to investigate how cisplatin affects the cellular hierarchy and phenotypes of breast cancer cell lines.

Flow cytometric analysis of protein expression revealed a novel cellular hierarchy in the breast cancer cell lines examined which differed depending on the cell line. This was consistent with the previously suggested breast cancer heterogeneity (Clarke, Dick et al. 2006). The stem cell markers SSEA4 and CD49f were highly expressed in the more aggressive CSC-enriched and triple-negative cell lines, whereas the differentiation markers CK18 and β-tubulin were highly expressed in the more differentiated cell lines. It is surprising that the MDA-MB-468, a basal-like triple negative line, is more differentiated than other triple-negative lines, such as BT-549 and MDA-MB-231.

Cisplatin treatment shifted this cellular hierarchy towards more differentiated cells by selectively targeting and down-regulating the stem cell markers CD49f and SSEA4 by 50-70% in the more invasive breast cancer cell lines (BT-549 and MDA-MB-231), whilst up-regulating the differentiation markers CK18, SMA and β-tubulin by 10-130% (Fig. 2; Table 4). High CD49f and SSEA4 expression has been associated with low levels of tumour differentiation and reduced survival in breast cancer patients, and is often more prevalent in TNBCs (Zeidan, Jenkins et al. 2008; Stagg and Pommey 2009; Meyer, Fleming et al. 2010; Sanges and Cosma 2010). CD49f and SSEA4 along with other surface markers, such as CD24, CD44, CD133, has been commonly used for the
detection of CSCs in solid tumours, including human breast, brain, colon and ovarian cancer (Zhou, Zhang et al. 2009), as well as for categorising breast cancer molecular subtypes (Hergueta-Redondo, Palacios et al. 2008; Nakshatri, Srour et al. 2009; Stagg and Pommey 2009). In contrast, the differentiation marker CK18 is highly expressed in the normal mammary glands (more than 90% cells), and its loss has been correlated with high tumour grade (Woelfle, Sauter et al. 2004). These findings clearly show that cisplatin treatment influenced gene expression in the breast cancer cell lines tested to shift them towards a more differentiated phenotype.

The current anti-cancer drug therapies are undergoing a huge shift from cytotoxic-based to differentiation-inducing therapies, as many types of tumours acquire further resistance, recur and/or metastasise after treatment due to survival of the resistant CSCs (Prat, Parker et al. 2010). The general rule of tumour cell differentiation therapy is to force maturation of less differentiated CSCs or cancer progenitor cells into specific lineages, which in turn reduces proliferation capacity and tumorigeneicity (Hadnagy, Beaulieu et al. 2008). Based on our findings, we propose that cisplatin not only induces symmetrical differentiation, but also asymmetrical differentiation, shifting BT-549 cells towards a myoepithelial phenotype, and MDA-MB-231 and MDA-MB-468 cells towards a luminal phenotype. The fate of switching from one cell type to another (myoepithelial to epithelial cell type and vice-versa) may depend on the more dominating or prominent progenitor cell type present in each cell line. An interesting and recent study has shown that cisplatin triggered differentiation of CSC-enriched testicular embryonal carcinoma cells and in parallel induced cisplatin resistance (Abada and Howell. 2014). In the same study, the correlation between cisplatin mediated differentiation and resistance induction was tested in other cancer cells which were insensitive to cisplatin that resulted in no differentiation and cisplatin resistance (Abada and Howell. 2014). For the differentiation process to occur, various proliferation related genes such as SOX2, NANOG and OCT4 may be differentially affected which subsequently activates other targets of these genes which induce cisplatin acquired resistance. Therefore it is important to identify a highly potential drug that is able to completely diminish the persisting refractory cells within the tumour along with cisplatin in cancers that are solely reliable on this platinum drug. However, extensive study is required to further confirm the mechanism of cisplatin-mediated induction of differentiation. A possible mechanism of cisplatin-induced differentiation in breast cancer cells is direct interference of the cis-platinum compound with the DNA
sequence at the reactive nuclear sites, causing conformational changes and inhibiting transcription, which eventually leads to cancer cell death. This can be correlated with various levels of binding capacity to target sites in the nuclear DNA of the tumour cell. Even partial binding of cisplatin to the DNA sequence, 1,3 intrastand GpG crosslink, or other unknown crosslink patterns, can trigger changes in gene expression and cell function. A second mechanism of cisplatin action could involve binding of the cis-platinum compound to non-DNA targets. This is supported by a previous study demonstrating that less than 1% of the cis-platinum compound binds and form adducts with the nuclear DNA of the tumour cell, while 75-85% of it forms covalent bonds with thiol peptides, proteins, RNAs and other cellular constituents (Cepeda, Fuertes et al. 2007). Other studies have also shown that platinum compounds can bind to cellular proteins such as the zinc-fingers, tubulin as well as actin (Cepeda, Fuertes et al. 2007; Wexselblatt, Eylon et al. 2012). Consistent with this, we show that cisplatin clearly affects the cytoskeleton through morphological changes as well as clear changes in the cytoskeleton proteins β-tubulin, CK18 and SMA (Fig. 3 and Fig. 4). Binding of cisplatin to these proteins may lead to structural conformation changes that restrict movement of cell proliferation-controlling transcription factors to the nucleus and attachment to their target promoters (Nguyen, Almeida et al. 2010). Fig. 7 illustrates a proposed model based on our findings which depicts the role of cisplatin in differentiation of breast cancer cells.

**Figure 6. A proposed model of cellular hierarchy in breast cancer cells and how it is influenced by cisplatin.** The diagram illustrates a possible mechanism of cisplatin effects in breast cancer cells. Cancer stem cells can differentiate, potentially through a progenitor step, into a more mature epithelial phenotype, luminal or myoepithelial, which has lost or possesses limited proliferative potential. As the more differentiated cells are more susceptible to chemotherapy, this push of differentiation may assist in the management and/or treatment of breast cancer.
In addition to effects of cisplatin at the protein level, we demonstrated differential gene regulation of cisplatin at the mRNA level. CD49f and β-tubulin mRNA and protein expression were affected in a similar way by cisplatin in BT-549 and MDA-MB-231 cells, suggesting that cisplatin’s point of interference was at the DNA sequence. However, SSEA4 expression was influenced differently at the mRNA and protein levels by cisplatin treatment, with decrease in protein expression, but an increase in mRNA expression. This suggests that this drug may influence certain genes differentially at the post-transcriptional or post-translational levels without affecting the DNA sequence, which could be linked to epigenetic regulation (Hadnagy, Beaulieu et al. 2008). This is further supported by a recent study showing that SSEA markers are among the most epigenetically accessible and can in turn mediate the expression of chromatin remodelling factors that are accountable for the principal epigenetic modifications (Sanges and Cosma 2010). Taken together, these results suggest that in the tumour environment cisplatin may activate binding of other components to DNA, resulting in disruption of DNA transcription.

3.6 Conclusions
Breast tumours are characterised by a cellular hierarchy, similar to the healthy resting and lactating breast (Hassiotou, Beltran et al. 2012), containing cancer stem cells and more differentiated cancer cells, each with different susceptibility towards various anti-cancer drugs. Cisplatin seems to shift this hierarchy towards more differentiated cells that are less proliferative. Although the mechanism of action of cisplatin still remains elusive, we demonstrated differential effects at the mRNA and protein levels for some genes, suggesting involvement of both or either of epigenetic mechanisms and recruitment of other cellular components that influence gene transcription and/or translation. Further studies are needed to give insight into how cisplatin acts on tumour cells, and whether and how it may influence their normal cell counterparts. Therefore it is important to identify a highly potential drug that is able to completely diminish the persisting refractory cells within the tumour along with cisplatin in cancers that are solely reliable on this platinum drug.
CHAPTER 4

EN1 TARGETING VIA INTERFERENCE PEPTIDE (iPEP) TECHNOLOGY REGULATES INFLAMMATORY GENE EXPRESSION IN TRIPLE NEGATIVE BREAST CANCER
EN1 Targeting via interference Peptide (iPep) Technology controls inflammatory gene expression in triple negative breast cancer (TNBC)

4.1 Abstract
Increasing studies have shown that resistance of CSCs to therapy is linked to a dysfunctional immunoresponse and/or overexpression of pro-inflammatory mediators such as cytokines, chemokines and growth factors that sustain angiogenesis, tissue invasion and metastasis in the tumour environment. Thus, recent efforts are focusing on identifying treatments that may resolve inflammation in inflammation-related cancers, which are very aggressive and lethal. A recent study has shown the overexpression of a neural-specific TF, EN1 in TN-IBCs. In this study, the mechanism of action of an EN1 iPep that inhibits EN1 TF function and resolves inflammation in TN-IBCs was investigated. Immunofluorescence microscopy demonstrated a specific nuclear as well as perinuclear localization of the iPep and its uptake induced apoptosis in SUM-149 cells, which represents the TN-IBC. Viability assays (MTS) were conducted in two highest EN1-expressing cell lines, the SUM-149 breast cancer and T11 mouse mammary carcinoma cell lines which were treated with an active (Ca-520) and mutant (Ca-521) EN1 iPep respectively for 8 hours at 0-50 µM. This assay revealed IC\textsubscript{50} values of 10.10 µM and 13.32 µM in SUM-149 and T11 respectively, while a mutant EN1 iPep did not cause any harm to the cells. In addition, cisplatin, an important TNBC chemotherapy drug was also administered using similar concentrations, whereby IC\textsubscript{50} values of 18.35 µM and 13.23 µM were achieved respectively. Interestingly, the active iPep did not synergize with cisplatin in a combinatorial treatment. Furthermore, the EN1 iPep also showed a co-localization with EPRS, an important protein involved in the transcript specific translational control of inflammatory proteins. Radioactive labelling of proline and methionine incorporation assay revealed a significant reduction, which could be due to the interaction of EN1 iPep with EPRS, while addition of proline amino acid in excess (50 µM) re-established cell viability. This suggested abrogation of protein synthesis caused mainly by the lack of proline and leading to amino acid starvation. Lastly, gene expression of a variety of inflammatory targets such as VEGFA, COL1A1, CD69, IL-11 along with EPRS, EN1 and FOXA2 were examined in mutant and active EN1 iPeps treated SUM-149 cells at 2, 6 and 10 hours, along with non-treated cells, dsRED (ShRNA-mediated knockdown of EN1) and EN1 cDNA expressing SUM-149 cells as the controls. A significant down-regulation of pro-inflammatory (CD69 and COL1A1) and up-regulation of anti-inflammatory (IL-11) as well as tumour
suppressor (*FOXA2*) genes were observed in the EN1 active iPep treated cells. In conclusion, the EN1 iPep interfered with the prime function of *EN1* TF and EPRS in SUM-149 TN-IBC potentially by activating the amino acid response (AAR) pathway and controlling regulation of inflammatory genes. The EN1 iPep as a specific TF inhibitor may be an important therapeutic tool in treating *EN1* expressing cancers and potentially other immuno-compromised diseases.

4.2 Introduction

TNBCs are aggressive with no appropriate targeted therapies as this cancer subtype lacks the ER, PR and HER2 receptors (Hartman, Poage et al. 2013). The TNBCs are CSC-enriched which often leads to poor prognosis and worse overall survival rate in patients (Bosch, Eroles et al. 2010; Hastak, Alli et al. 2010; Tiwary, Yu et al. 2011) due to high proliferative and metastatic capacity of the cancer cells (Bosch, Eroles et al. 2010; Hastak, Alli et al. 2010; Tiwary, Yu et al. 2011). In addition to that, some TNBCs are also IBC which account for 2.5% of all breast cancer cases (Robertson, Bondy et al. 2010) making the TN-IBC cancer the most aggressive and resistant breast cancer type. This is often a lethal disease with less that 5% survival rate (Robertson, Bondy et al. 2010). The platinum-based drug cisplatin is one of the most effective drug used in treating TNBCs for the past 4 decades (Basu and Krishnamurthy 2010; Pines, Kelstrup et al. 2011) apart from poly (ADP-ribose) polymerase (PARP) inhibitors and taxol, a mitosis inhibitor (Bosch, Eroles et al. 2010; Hastak, Alli et al. 2010; Higgins and Baselga 2011). However, not much is known about the specific cisplatin effect mechanisms in IBCs. In the previous chapter, findings show that cisplatin induced differentiation of CSC-enriched TNBCs (Prabhakaran, Hassiotou et al. 2013).

Increasing studies have shown that resistance of CSCs to therapy is linked to a dysfunctional immunoresponse and/or overexpression of pro-inflammatory mediators such as cytokines, chemokines and growth factors that sustains angiogenesis, tissue invasion and metastasis in the tumour environment (Standiford 2000; Grivennikov, Greten et al. 2010; Putoczki and Ernst 2010; Robertson, Bondy et al. 2010; Beltran, Graves et al. 2013). These inflammatory responses play vital roles in tumour cell development especially in the initiation, promotion and metastasis (Grivennikov, Greten et al. 2010). About 20% of cancers are associated with chronic inflammation such as the colorectal cancer through the inflammatory bowel disease (IBD) (Grivennikov, Greten et al. 2010); gastric cancer which is initiated by *Helicobacter pylori* infection (Mantovani, Marchesi et al. 2008); and cancer of the liver through Hepatitis B infection...
The most commonly found immune cells within the tumour microenvironment are tumour-associated macrophages (TAMs) and T cells (Roberts, Ng et al. 2007; Grivennikov, Greten et al. 2010). As inflammation is considered to be a tumorigenesis enhancer, there is a great need to identify targeted therapies, which could induce inflammatory resolution.

A recent study by the Blancafort laboratory has demonstrated a selective overexpression of a neural-specific transcription factor (TF), ENGRAILED-1 (EN1) in TN-IBC (Beltran, Graves et al. 2013). EN1 and EN2 are two important paralogues and members of the homeobox gene family essential in the early embryonic developmental stage in the central nervous system (CNS) whereby it controls cell identity, cell growth and differentiation (Beltran, Graves et al. 2013; McGrath, Michael et al. 2013). The overexpression of EN1 has been associated with tumour development in adults, especially breast, prostate, melanoma and ovarian cancers (Beltran, Graves et al. 2013; McGrath, Michael et al. 2013) by promoting survival of tumour cells and resistance towards chemotherapy. The significant pro-oncogenic role of EN1 suggests EN1 as a potential diagnostic biomarker in cancer. This homeodomain TF (TF\textsubscript{HD}) EN1 is known to interact with other TF\textsubscript{HD}s and other regulators whereby the protein-protein (TF\textsubscript{HD}-TF\textsubscript{HD}) interaction is essential for cooperative DNA binding in order to activate the oncogenic activity (Beltran, Graves et al. 2013; McGrath, Michael et al. 2013). *HOXA9* and *PBX1* are important TF\textsubscript{HD}s and binding partners EN1 (Beltran, Graves et al. 2013; McGrath, Michael et al. 2013). The Blancafort laboratory engineered synthetic interference peptides (iPeps) comprising of EN1-specific sequences that blocked the essential protein-protein interactions, consequently inhibiting EN1 oncogenic function in EN1 overexpressing SUM-149 TN-IBC cells (Beltran, Graves et al. 2013). They also discovered and demonstrated a new and important EN1 target, the glutamyl-prolyl tRNA synthetase (EPRS) (Beltran, Graves et al. 2013) an aminoacyl-tRNA synthetase (aaRS) and a housekeeping enzyme that ligates glutamine (Glu) and proline (Pro) amino acids to specific tRNAs as an essential process in protein synthesis (Ray and Fox 2007; Brown, Reader et al. 2010; Keller, Zocco et al. 2012).

The aaRS are known to have versatile functions in transcription, translation, splicing, inflammation, angiogenesis and apoptosis (Brown, Reader et al. 2010). EPRS is also an important element of the IFN-\gamma-activated inhibitor of translation (GAIT) complex that is involved in transcript specific translational control of inflammatory proteins (Mukhopadhyay, Jia et al. 2009; Brown, Reader et al. 2010; Arif, Jia et al. 2011; Jia, Yao et al. 2013) and activation of amino acid stress pathways (Keller, Zocco
et al. 2012; Beltran, Graves et al. 2013). EPRS Halofuginone (HF), a febrifugine derivative was recently identified as EPRS inhibitor, mainly by inhibiting the proly-tRNA synthetase activity which eventually triggered the amino acid response (AAR) pathway due to amino acid starvation (Sundrud, Koralov et al. 2009). However, not much is known about EPRS’ role in tumorigenesis although overexpression of another aaRS, the methionine (MRS) have been detected in colon cancer, malignant fibrous histiocytomas, sarcomas, gliomas and glioblastomas (Kim, You et al. 2011). In addition to the GAIT complex, EPRS is also a member of the multisynthetase complex (MSC) which is an crucial machinery for protein synthesis in which it binds to three non-enzymatic factors AIMP1, AIMP2 and AIMP3 which are involved in various regulatory pathways in tumorigenesis (Kim, You et al. 2011; Guo and Schimmel 2013; Kim, Park et al. 2013). The AIMP3 factor is important in the regulation of tumorigenesis whereby depletion of this factor has shown to increase proliferation and survival in mice and has been linked with spontaneously development of lymphoma, breast cancer, bronchiole epithelium adenocarcinoma and hepatocarcinoma while increased AIMP3 expression induced cell senescence (Kim, You et al. 2011; Guo and Schimmel 2013). However, the role of the MSC components and EPRS in cancer is still unclear. Increased intracellular AIMP3 levels cause a translocation to the nucleus which leads to $p53$ activation in the presence of DNA damage (Mukhopadhyay, Jia et al. 2009; Kim, You et al. 2011; Guo and Schimmel 2013). Aberrant function of a MSC aaRS could possibly influence its interaction with AIMP3 and may actually modulate the tumour suppressive activity in the nucleus. It is known that apoptosis signal-regulating kinase 1 (ASK1) is usually activated by various oncogenic stresses (Park, Schimmel et al. 2008).

Most interestingly, the EPRS links both the MSC complex and GAIT complex with specific functions in protein synthesis by which EPRS aminoacylation in MSC supports global translation while translocation to the GAIT complex causes inflammatory gene specific translational silencing (Fig. 1). Based on all these previous studies, it can be concluded that the breast cancer disease involves various pathways in regulating different activities, such as inflammation and protein synthesis in order to sustain tumour cell survival and longevity. As EN1 reveals as an important activator of intrinsic inflammatory pathways linked to longevity in TN-IBC through the binding of EPRS, this chapter mainly revolves in the further investigation of the EN1 iPep in resolving inflammation in breast cancer.
Figure 1. EPRS as an important component of protein synthesis. IFN-γ-induced release of EPRS from the MSC and binding of the GAIT complex which mediated translational silencing of inflammation-related mRNAs. Adapted from (Yao and Fox 2013).

4.3 Materials and Methods

4.3.1 Cell Culture

SUM-149 breast cancer cells and T11 mouse mammary carcinoma cells (American Type Culture Collection, ATCC) (Table 1) along with dsRED SUM149 (lentivirally-delivered shRNA) and EN1 flag SUM149 (lentivirally-delivered EN1 cDNA) breast cancer cells which were developed in the Blancafort laboratory (Chapel Hill NC, USA) were cultured in T25 flasks (Corning, Tewksbury MA, USA) at 37°C and 5% CO₂.

SUM149 related cells were cultured in F-12 (1X) Nutrient Mixture (Ham) medium (Invitrogen, Carlsbad, CA, USA), supplemented with 5% FBS (Serana, WA Pty Ltd, Bunbury, WA, Australia), and 1% antibiotic-antimycotic (100 U/mL penicillin, 100 μg/0.25μg/mL streptomycin) (Invitrogen) while T11 cells were cultured in RPMI Medium 1640 (RPMI + Glutmax™1(1X)) (Invitrogen) supplemented with 10% FBS and 1% antibiotic-. Cells were passaged twice a week, week at 60-70% cell confluency.

4.3.2 Determination of Cell Viability

Cell cultures of 60-70% confluency were used for experiments. A 100-μL of 3×10³ cells supplemented with 5% FBS and 1% antibiotic-antimycotic per well was seeded in flat
bottom 96-well plates (Sarstedt, Newton, USA). This condition is used for all experiments unless after 24 hours, cisplatin (Sigma-Aldrich, St Louis, MO, USA), Ca-520 and Ca-521 iPeps (ChinaPeptides Co., Ltd., China) were each directly added at different concentrations (2.5, 5, 10, 15, 20, 25 and 50 µM). These concentrations were chosen according to the range of cisplatin concentrations used in treatments (Persons, Yazlovitskaya et al. 1999; Zhang, Gao et al. 2003; McAuliffe, Morgan et al. 2012). Cells were incubated with the EN1 iPeps for 8 hours respectively (as was used in a previous study by the Blancafort laboratory) while cisplatin for 24 hours. As for the combination treatment, a fixed concentration of 5 µM EN1 iPep was added 2 hours prior to addition of various cisplatin concentrations (2.5, 5, 10, 15, 20, 25 and 50 µM), and were incubated for 8 hours in total. The 5µM EN1 iPep concentration was chosen mainly because it was low and was able to reduce cell viability significantly while various cisplatin concentrations were used to identify an effective low cisplatin dose, which may induce a significant anti-cancer effect in synergy with the iPep. Furthermore, the iPep was added first as a preventive step to prevent peptide from being digested by DMSO in which cisplatin is dissolved. The cell viability was assessed by MTS colorimetric assay, using Cell Titer 96® Aqueous (Promega, Madison, USA), according to the manufacturer’s instructions. The MTS substrate was added (10-µL in 100-µL medium) and incubated for 4 hours. Finally the MTS activity is read at 490 nm using a plate reader (Labsystems Multiskan RC). The higher the the number of viable cells, the higher is the purple color intensity. A 50% reduction in cell viability (IC$_{50}$) of each drug treatment was calculated using the GraphPad Prism 6 software. Experiments were done in quadruplicate in three independent experiments.

### 4.3.3 Immunofluorescence Microscopy

Cells (3×10$^4$ cells per mL) were grown on glass coverslips in 24-well plates (Sarstedt) for 24 hours. 15 µM of fluorescence tagged active/wild type (P871) and mutant (P872) EN1 iPeps synthesized by the UNC High-Throughput Peptide Synthesis and Array Facility at UNC (Chapel Hill, North Carolina, USA) were used. In iPep internalization microscopy, SUM-149 cells were incubated with each iPep respectively for 2 hours in serum-free medium to eliminate interference between the iPeps and FBS, followed by fixation with 1% PFA in PBS/2% sucrose, permeabilised with 0.1% Triton X-100 in PBS for 30 minutes, DAPI (Roche, 1:100) nuclear staining for 10 minutes and finally coverslips are transferred onto glass slides. Cells on the coverslips are washed (2x) with PBS at each step. In contrast, EPRS-EN1 iPep co-localization was done by treating cells
CHAPTER 4: EN1 IN INFLAMMATORY BREAST CANCER

with the respective for 2, 6 and 10 hours in the similar conditions as mentioned previously in section 4.3.2. Cells were then fixed with 1% PFA in PBS/2% sucrose, permeabilised with 0.1% Triton X-100 in PBS for 30 minutes, incubated overnight with EPRS (Sigma-Aldrich, St Louis, MO, USA), and then incubated for 4 hours with secondary antibody (Alexa Fluor 594 goat Anti-Rabbit IgG (H+L) (Invitrogen, USA) and DAPI (Roche, 1:100) for nuclear staining. Appropriate negative controls (secondary antibody only) were used. Coverslips with cells were then transferred to glass slides with fluorescence mounting medium (Dako) and sealed with colourless nail polish. Cells were imaged using an upright Nikon Eclipse 90i microscope.

4.3.4 Determination of Caspase-3 Processing
SUM-149 breast cancer cell cultures of 60-70% confluency were passaged by trypsinisation. A day later, the cells were treated with 10 μM active and mutant EN1 iPeps respectively and incubated for 8 hours. Cells were then gently scraped and centrifuged at 145g for 5 minutes. Protein extraction was done using the radioimmunoprecipitation assay (RIPA) buffer in which the final supernatant (protein) collected was further used for protein quantification (Bradford assay), followed by apoptosis detection using the Human Caspase-3 (active) colorimetric assay (Invitrogen ELISA Kit, Catalog# KHO1091, Carlsbad, CA, USA), according to the manufacturer’s instructions. Appropriate standards and cell extracts were incubated at room temperature for 2 hours in the caspase-3 antibody coated wells. Next, the wells were incubated with detection antibody for an hour and followed on by Horse Radish Peroxidase (HRP) anti-rabbit antibody incubation for 30 minutes. This step is then continued with further 30 minutes incubation with stabilized chromogen and finally a stop solution is added. Reading is taken using a plate reader at 450nm. The higher the active caspase-3 activity in samples, the higher was the blue colour intensity. Each solution in samples was aspirated and wells were washed prior to each step.

4.3.5 Radioactive Proline/Methionine Incorporation Assay
SUM-149 breast cancer cell cultures of 60-70% confluency were passaged by trypsinization. Cells were seeded at a density of 5X10^4 per mL. A day later, the SUM-149 cells were treated with 10 μM Ca-520 iPep Untreated/mock SUM-149 cells was used as controls. Target cells were incubated with L-[2,3-3H]-Proline / L-[Methyl-3H]-Methionine at 1 mCi /ml of media, at 37°C/5% CO₂ for 4hrs. Subsequently, SUM-149 cells were treated with the Ca-520 iPep & incubated for 4hrs at 37°C/5% CO₂ in Tissue
Culture Suite 233A. Following peptide incubation, cells were pelleted & washed, retaining aspirated media. Liquid scintillation cocktail was added to the cell pellets & media and read for 10 minutes on the liquid scintillation.

4.3.6 Proline Rescue Assay

Cell cultures of 60-70% confluency were used for experiments. A 100-µL of $3 \times 10^3$ cells supplemented with 5% FBS and 1% antibiotic-antimycotic per well were seeded in flat bottom 96-well plates (Sarstedt, Newton, USA). After 24 hours, a fixed concentration of 5 µM aCa-520 iPep (ChinaPeptides Co., Ltd., China) along with L-proline (Sigma-Aldrich, St Louis, MO, USA) at different concentrations (1, 2.5, 5, 10, 15, 20, 25 and 50 µM) were added directly and simultaneously into cells per well and incubated for 8 hours before the proline rescue on cell viability was assessed with the MTS colorimetric assay, using Cell Titer 96® Aqueous (Promega, Madison, USA). Similar experiment was done using methionine as a control. The MTS substrate was added (10-µL:100-µL medium) and incubated for 4 hours. Finally the MTS activity is read at 490 nm using a plate reader (Labsystems Multiskan RC). The experiments were done in quadruplicate in three independent experiments.

4.3.7 mRNA Quantification by TaqMan qRT-PCR

Cells were seeded and treated using similar protocols as found in Chapter 3 (Heading 3.3.5). Total cellular RNA was extracted using RNAzol®RT (Molecular Research Center, Inc). RNA quantity and quality were assessed with NanoDrop 1000 (NanoDrop, Wilmington, DE). For each sample, 5 µg RNA was treated with RQ1 RNase-Free DNase (Promega, Madison, USA) and was reverse transcribed using MMLV (Promega) by incubating at 25°C (10 minutes), 55°C (50 minutes) and 70°C (15 minutes) using PTC-100™ Programmable Thermal Controller (MJ Research Inc.). The RT Reaction Clean-Up MoBio kit (MoBio Lab Inc., CA, USA) was used for cDNA clean up. The qRT-PCR reaction was performed with TaqMan Fast Universal Master Mix (Applied Biosystems, Carlsbad, CA). A 10-µL reaction mixture per sample which consisted of 5-µL Master Mix, 0.5-µL of primer for each gene (Table 1), 3.66-µL of RNAse free H2O and 0.84-µL of cDNA was used to detect the relative abundance of transcripts. The conditions for all qRT-PCR reactions were as follows: 20 minutes at 95°C (holding stage) followed by 3 seconds at 95°C and 30 seconds at 60°C (cycling stage) for 40 cycles. GAPDH was used as the internal positive control/housekeeping gene and non-template sample as the negative control. A comparative $C_T$ ($\Delta \Delta C_T$) was used to analyse the mRNA expression.
CHAPTER 4: EN1 IN INFLAMMATORY BREAST CANCER

TABLE 1: TaqMan primers used for RT-PCR.

<table>
<thead>
<tr>
<th>Gene</th>
<th>Specification</th>
<th>Catalogue No.</th>
</tr>
</thead>
<tbody>
<tr>
<td>GAPDH</td>
<td>Hu GAPDH (20X) FAM MGB</td>
<td>4333764-1207037</td>
</tr>
<tr>
<td>EPRS</td>
<td>Hs00164004_m1 EPRS</td>
<td>634775 D12</td>
</tr>
<tr>
<td>VEGFA</td>
<td>Hs00164004_m1 VEGFA</td>
<td>1208393 B8</td>
</tr>
<tr>
<td>COL1A1</td>
<td>Hs00164004_m1 COL1A1</td>
<td>1078470 B3</td>
</tr>
<tr>
<td>FOXA2</td>
<td>Hs00232764_m1 FOXA2</td>
<td>1204538 B1</td>
</tr>
<tr>
<td>CD69</td>
<td>Hs00934033_m1 CD69</td>
<td>1216392 F7</td>
</tr>
<tr>
<td>IL11</td>
<td>Hs01055413_gl IL11</td>
<td>1189540 H6</td>
</tr>
<tr>
<td>EN1</td>
<td>Hs00154977_m1 EN1</td>
<td>1215969 A12</td>
</tr>
</tbody>
</table>

4.3.8 Statistical Analysis

Statistical analysis and graphical exploration of the data were done in Microsoft Excel. The Student’s paired t-test with a two-tailed distribution was used to compare cisplatin-treated and untreated breast cancer cells. The results are presented as mean±SEM (MTS and qRT-PCR), as indicated in the corresponding figure legends. The significance is shown as follows: *p≤0.05; **p≤0.01; ***p<0.0001.

4.4 Results

4.4.1 EN1 interfering peptide (iPep) structure and perinuclear/ nuclear localization

To halt the EN1 TF function in basal-like breast cancer cells, a synthetic interference peptide (iPep) was engineered and established. The Ca-520 comprised EN1-specific hexamotif sequence as well as a flanking protein sequence on the N-terminus of the homeodomain (HD) (Fig. 2A). The hexamotif sequence (WPAWVY) which is found in the EN1 protein allows binding and interaction with its counterparts, mainly TF\textsubscript{HD} that activates the oncogenic activity through cooperative DNA-binding (Beltran, Graves et al. 2013). This EN1 iPep is designed in a way that mimics the highly conserved protein-protein surfaces which prevents the TF\textsubscript{HD} - TF\textsubscript{HD} interaction, inhibiting the oncogenic activity by inducing selective cell death. Along with the active EN1 iPep, the Ca-521 iPep was also established as a positive control (Fig. 2A). A double mutation on the hexamotif sequence was made by substituting both the Tryptophan (W) amino acid with
Alanines (A) (Fig. 2A). Apart from that, a specific cell penetrating peptide (CPP) sequence (KKKRVK), which is also a nuclear localization sequence (NLS) was included in the N-terminus of the i Pep sequence variants (Fig. 2A). This particular NLS/CPP sequence has been shown to effectively mediate penetration of peptide cargos containing W and Y residues that are hydrophobic in nature (Morris, Deshayes et al. 2008). In order to confirm that the peptides are taken up into cells, peptide internalization studies were performed. Both the active and mutant iPeps were coupled to a C-terminal fluorescein molecule (P871 and P872 respectively) and delivered into SUM-149 breast cancer as these cells are known to express high levels of EN1 (Beltran, Graves et al. 2013). Cells were imaged after two hours using the upright Nikon Eclipse 90i microscope. The Ca-520 i Pep was taken up within 2 hours and showed specific nuclear as well as perinuclear localization while the Ca-521 i Pep showed a more non-specific nuclear and cytoplasmic localization (Figure 2B). Furthermore, with the use of Flex pep dock software (Li, Lu et al. 2013), a preliminary binding pattern of the active i Pep on EPRS was studied by keying in the peptide sequence (Fig. 2C). It is known that the halofuginone drug, an EPRS inhibitor binds directly in the binding pocket of EPRS (Son, Lee et al. 2013) and similarly the Ca-520 i Pep showed high tendency for the same binding pocket. The i Pep bound firstly on the surface of the pocket and what was more interesting is that the amino acid proline (P) on the hexamotif (WPAYVY) sequence initiated a kink and pushed the hexamotif sequence directly into the binding pocket while the CTRYSDR sequence remained on the surface by forming a cap on the binding pocket of EPRS (Fig. 2C).
Figure 2. EN1 interfering peptides (iPeps) sequence, cellular uptake and localization. (A) Active EN1 iPep (Ca-520 & fluorescence tagged P871) containing the highly conserved hexamotif sequence while mutant EN1 iPep (Ca-521 & fluorescence tagged P872) sequence comprising of double Tryptophan (W) mutation, substituted with Alanine (A). (B) Cytoplasmic, perinuclear and nuclear internalization of EN1 interfering peptides (iPeps) in SUM149 cells. Cells were incubated with 15 µM active/wild type (wt) EN1 iPep (P871) and mutant EN1 iPep (P872) for 2 hours respectively. (C) Active EN1 iPep binding on the glutamyl-prolyl tRNA synthase (EPRS) using the Flex pep dock software whereby the structure is distinguished by the colors, green: EPRS; red: active EN1 iPep; pink: Halofuginone. Figures A and C were designed and constructed by Pilar Blancafort.

4.4.2 EN1 iPep inhibits cell viability of breast cancer cells by inducing apoptosis

Based on the EN1 gene expression, a series of human and mouse breast cancer cells (Fig. 3A and 3C), and also SUM-149 basal-like breast cancer and T11 mouse mammary carcinoma cells representing highly invasive TNBCs, the EN1 iPep was further studied in combination with cisplatin. TNBCs have shown great sensitivity towards cisplatin treatment in patients that undergo chemotherapy (Bosch, Eroles et al. 2010; Hastak, Alli et al. 2010; Tiwary, Yu et al. 2011). Both drugs showed a dose-dependent effect on T11 and SUM-149 cancer cells. The iPep was administered in these cells at increasing concentrations in a range of 0-50 µM. Treated cells were first analysed using the MTS assay, which monitors metabolic viability. The Ca-520 iPep significantly inhibited cell viability beginning from 5 µM concentration between 20-30% (p<0.0001), followed by
IC$_{50}$ values at 10.10 µM and 13.32 µM and about 90% (p<0.0001) at 50 µM in SUM-149 and T11 respectively. Interestingly, the Ca-521 iPep somewhat enhanced cell viability in both cell lines, though this was not significant and caused no harm to the cells up to 50 µM concentration (Fig. 3B and 3D).

Cisplatin treatment alone caused a slight insignificant increase in cell viability at the lower concentrations, while significantly reduced cell viability at 10 µM (↓20%, p<0.0001), followed by an IC$_{50}$ at 18.35 µM and a further reduction up to 80% (p<0.0001) at 50 µM in SUM-149 cells (Fig. 3D). T11 cells were more sensitive to cisplatin at lower doses as a significant decline in cell viability was observed at 5 µM (↓20%, p=0.003), followed by an IC$_{50}$ at 13.23 µM and further reduction up to 80% (p<0.0001) at 50 µM.

As both the EN1 iPep and cisplatin showed potent cell viability inhibition, a combinatorial treatment of these drugs was examined in SUM-149 to see if the active EN1 iPep sensitized cisplatin treatment. As cisplatin is known to be lethal at doses above 20 µM, it is important to identify appropriate and effective combinatorial treatments that allow effective use of cisplatin at low concentrations to prevent severe toxicity to patients. Therefore, low cisplatin dose (5 µM) was used in combination with varying Ca-520 iPep doses in the hope of investigating a possible additive or synergistic effect which sensitized these cells to cisplatin via inhibition of EN1 expression. No synergistic effect was found between the drugs; instead, the active EN1 iPep appeared to obstruct the cytotoxic effect of cisplatin (Fig. 3D). Chapter 3 of this thesis revealed a new cisplatin mechanism of action, which demonstrated differentiation induction in breast cancer cells, especially in TNBCs (Prabhakaran, Hassiotou et al. 2013). In order to investigate the EN1 iPep mechanism of action, the EN1 iPep-treated SUM-149 cells were subjected to an active caspase-3 ELISA assay which showed clear apoptotic induction by a significant increase in active caspase-3 protein compared to vehicle-treated and mutant EN1 iPep-treated cells (Fig. 3E). These findings suggest that the cisplatin and the EN1 iPep each reduce cancer cell viability via different pathways that do not act synergistically to enhance cancer cell death. In contrast, these pathways may interact with each other in an opposite way, i.e. one may block the other, hence the reduction of cancer cell cytotoxicity of cisplatin treatment in the presence of the EN1 iPep. This merits further investigation.
Figure 3. EN1 overexpression and cell viability inhibition using cisplatin and active EN1 iPep in TNBC breast cancer cells. (A and C) EN1 mRNA levels by qRT-PCR in a panel of human and mouse breast cancer cell lines (these figures were adapted from (Beltran, Graves et al. 2013). (B) Effect of cisplatin, active/wt (Ca-520) and mutant (Ca-521) EN1 iPeps at increasing concentration (0, 2.5, 5, 10, 15, 20, 25 and 50 µM) on cell viability in T11 mouse mammary carcinoma cells. (D) Effect of cisplatin, active/wt (Ca-520) and mutant (Ca-521) EN1 iPeps in addition to (5 µM cisplatin + active EN1 iPep) combination at increasing concentration (0, 2.5, 5, 10, 15, 20, 25 and 50 µM) on cell viability in SUM149 breast cancer cells. Cell viability was determined by MTS assay. Cells were seeded at $3 \times 10^3$ density and incubated with the respective treatments; EN1 iPeps (8 hours), cisplatin (24 hours) and combination (8 hours). Experiments were done in quadruplicate in 3 independent experiments. The sigmoid graphs show the IC$_{50}$ (50% reduction in cell viability) value of each treatment in T11 and SUM149 cells. (E) Effect of 10 µM active EN1 iPep treated SUM149 cells on the production of active caspase-3. Active EN1 iPep treatment induced apoptosis in SUM149 cells.

4.4.3 EN1 iPep-EPRS co-localization potentially involved in the regulation of inflammation
The significant programmed tumour cell death induced by the active EN1 iPep and the fact that this iPep binds to EPRS, a glutamyl-prolyl tRNA synthase, shown in a recent study in our laboratory (Beltran, Graves et al. 2013), lead to further investigation of the
EN1 iPep- and EPRS protein-protein interaction in SUM-149 breast cancer cells. The expression of EPRS and the possibility of EN1 iPep-EPRS co-localization were examined at 2, 6 and 10 hour iPep incubation period (Fig. 4A). Cells treated with active EN1 iPep at 2 hours demonstrated highest EPRS expression followed by vehicle treated and mutant iPep treated cells (Fig. 4A). The expression level of EPRS remained the same in the vehicle treated cells at all time-points (Fig. 4A). A clear and specific co-localization with the active EN1 iPep was seen in the nucleus as well as in the perinuclear area, indicated by the bright yellow spots (Fig. 4A). Both EPRS and the active EN1 iPep expression in cells were down-regulated at 6 hours incubation (Fig.4A). At 10 hours incubation, EPRS expression was almost absent (Fig. 3A) In addition to that, another observation is that the active iPep (6 and 10 hours) and the iPep-EPRS co-localisation (6 hours) were localised more in the perinuclear area, and less in the nucleus (Fig. 4A). Expression of the active EN1 iPep decreased at 6 hours and was almost diminished at 10 hours, which may be due to degradation. In contrast, the EPRS showed a small degree of perinuclear co-localization with the mutant EN1 iPep at 2 hours incubation, while almost none at 6 and 10 hours (Fig. 4A).

The major role of EPRS in protein synthesis is to load two specific amino acids, namely glutamine and proline to its cognate tRNA. It is predicted that the co-localization of EPRS-EN1 iPep would interfere with protein synthesis possibly through the disrupted loading of the amino acids. Based on a previous study on a EPRS inhibitor (Keller, Zocco et al. 2012), which showed proline as a major target, a proline incorporation assay was conducted to check the level of proline before and after EN1 iPep treatment in SUM-149 in which methionine was used as a control. Proline incorporation activity was the highest in the mock/untreated cells, followed by the least in active EN1 iPep treated cells (Fig. 4B). In contrast, methionine incorporation was not affected or decreased upon active EN1 iPep treatment (Fig. 4B).
Figure 4. EN1 iPep-EPRS co-localization reduced proline production inducing cell apoptosis. (A) Co-localization of active/(wt) and mutant EN1 iPeps in SUM-149. Cells were incubated with 10 µM of iPep respectively at 2, 6 and 10 hours. (B) Proline incorporation assay: SUM149 cells treated with 10 µM of active EN1 iPep. (C) Proline rescue assay. Proline addition in excess to active EN1 iPep treated SUM-149 cells increased cell viability while methionine (control) caused no changes.

However, Fig. 4C shows that addition of proline substrate in excess to the active iPep treated cells increased cell viability once again in parallel to the mock. At 50 µM concentration, proline up-regulated cell viability from 70% in 5 µM active iPep-treated SUM-149 cells to 135%, while a further increase of viable cells was observed in the mutant iPep-treated cells (Fig. 4C). Methionine was used as a control, and as expected excess addition of methionine did not increase cell viability in both EN1 iPep treated SUM-149 cells. This finding indicates that proline amino acid biosynthesis is crucial for EPRS protein synthesis. The iPep may compete with proline, potentially through EPRS. Proline can rescue the cellular proliferation defect caused by the peptide.
4.4.4 EN1 iPep differentially regulates gene expression of inflammatory associated targets

To examine the role of the EN1 iPep in gene regulation of inflammatory targets possibly through the EN1-EPRS binding, VEGFA, COL1A1, CD69 and IL-11 along with EN1 and FOXA2 a tumour suppressor mRNA expression levels were measured in 10 µM active and mutant EN1 iPeps-treated SUM-149 cells respectively at 2, 6 and 10 hour time points. Appropriate control samples such as non-treated, dsRED (EN1 ShRNA knocked-down) and EN1 flag (EN1 cDNA inserted) SUM-149 cells were used to compare the mRNA expressions prior to and after iPep treatment with all conditions normalized to the mock/non-treated condition.

![Figure 5. Effects of EN1 iPep on mRNA expression of downstream targets in SUM149 breast cancer cells.](image)

*Figure 5. Effects of EN1 iPep on mRNA expression of downstream targets in SUM149 breast cancer cells.* Individual PCR reactions were normalised to a housekeeping gene (GAPDH) while all conditions are normalised to mock treated cells and plotted as the mRNA relative expression for dsRED cells (- EN1 control), EN1 flag cells (+ EN1 control), mutant and active EN1 iPep-treated cells. Cells were incubated with 10 µM active and mutant EN1 iPeps at 2, 6 and 10 hours respectively. Experiments were done in triplicates in 3 independent experiments. Bars are presented as mean±SEM. *p<0.05, **p<0.01 and ***p<0.0001.


EN1 was significantly up-regulated in the EN1-flag cells ($p<0.0001$) while it was significantly knocked-down in the dsRED cells, which suggest that these cells can be used as good controls. As a consequence of high EN1 expression in the flag cells, COL1A1 ($p<0.0001$), CD69 ($p<0.0001$), VEGFA ($p<0.0005$) and EPRS ($p<0.05$) were highly expressed compared to the dsRED SUM-149 cells (Fig. 5) indicating pro-inflammatory characteristics. CD69 is an activator of T cells and often correlates with chronic inflammation when highly expressed (Sancho, Gomez et al. 2006; Martin and Sanchez-Madrid 2011; Lozano, Joller et al. 2013), while VEGFA is a well-known vascular angiogenic mediator (Wakasugi, Slike et al. 2002; Ray, Jia et al. 2009; Keller, Zocco et al. 2012). In contrast, IL-11, an anti-inflammatory cytokine (Standiford 2000; Putoczki and Ernst 2010; Sultani, Stringer et al. 2012), and FOXA2, a tumour suppressor (Tang et al, 2012), were expressed at very low levels in the EN1-flag cells (Fig. 5).

Expression of these genes in the mutant EN1 iPep was similar to the mock cells, though an insignificant variation was seen in some genes expressed which could be due to the non-specific binding of the iPep in the nucleus. Most importantly, the active EN1 iPep treatment in SUM-149 cells significantly up-regulated IL-11 and FOXA2 by 7-8 fold in a similar pattern ($p<0.0005$ and $p<0.0005$ respectively) at 2 hours incubation and 3-6 fold ($p<0.0001$ and $p<0.0001$ respectively) at 6 hours incubation respectively, versus mutant EN1 iPep treated cells (Fig. 5). Interestingly at 10 hours incubation, both IL-11 and FOXA2 mRNA expression were decreased by 60% compared to 6 hours active EN1 iPep treatment, although the expression was still higher (3-6 fold), ($p<0.0005$ and $p<0.0001$ respectively) in comparison to the mutant iPep treated cells (Fig. 5). Apart from these two genes, a slight decrease at 2 hours incubation (about 9%, $p=0.023$) in CD69 mRNA expression was seen with the active EN1 iPep, followed by a further decrease up to 21% ($p<0.0005$) at 6 hours incubation and finally a complete inhibition (98%, $p<0.0001$) at 10 hours incubation in comparison to the mutant EN1 iPep-treated cells (Fig. 5).

The EN1 itself didn’t show any degree of inhibition at the gene level with the active iPep, instead the mRNA expression was intriguingly up-regulated versus the mutant iPep at 2 hours (219% ↑, $p<0.05$) (Fig. 5). However, the level of increase in mRNA expression compared to mutant iPep-treated cells declined gradually at 6 hours (67% ↑, $p=0.009$) and finally at 10 hours incubation the EN1 mRNA expression showed a negligible increase (6% ↑) (Fig. 5) which may suggest that either the EN1 expression
may be stationary or it may be further down-regulated after a 10 hours incubation. In addition to EN1, EPRS and its well-known target VEGFA showed similar expression pattern. The pro-inflammatory EPRS mRNA expression in the active iPep treated cells was always high (3 fold) with a slight increase at each time point from 2 hours to 10 hours incubation compared to the mutant iPep-treated cells. This finding was contrary to the EPRS protein expression pattern (Fig. 4A), which showed that EPRS expression decreased at 6 hours incubation and was almost completely inhibited at 10 hours with the active iPep, while the vehicle and mutant iPep treated cells showed persisting EPRS expression (Fig. 4). Next, VEGFA, an angiogenic-associated target was expressed higher (3 fold) than the mutant iPep treated cells at 2 hours and 6 hours, however a significant drop was observed from 6 hours to 10 hours active iPep incubation (52%) although the expression was still higher compared to the mutant iPep-treated cells (Fig. 5). In spite of that, COL1A1 which is enriched with proline and another EPRS target, when treated with active EN iPep showed a small decline at 2 hours (3%, \( p=0.032 \)), followed by a further 5% decline (\( p=0.012 \)) at 6 hours and finally 41% at 10 hours incubation (\( p<0.0005 \)) (Fig. 5). This suggests that the active EN1 iPep may differentially regulate gene expression, with some genes regulated at the transcriptional level, while others at the post-transcriptional level.

4.5 Discussion
Breast cancer is the most frequent cancer among women worldwide and the recently identified TNBC subtype is the most difficult to treat as there are no appropriate targeted therapies to date (Al-Ejeh et al. 2011). The TNBC subtype mostly consists of the basal-like and claudin-low subtypes which are highly aggressive breast cancers with very poor prognosis, whereby patients often have the worst survival rate (Tiwary, Yu et al. 2011). A recent study demonstrated a selective overexpression of EN1, a neural specific TF interestingly in basal-like breast cancers with the highest expression in SUM-149 cells (Beltran, Graves et al. 2014). The SUM-149 cell line is of the TNBC subtype and in addition, an IBC, which is a rare and aggressive form of invasive breast cancer. Expression of EN1 is believed to give defensive features to breast cancer cells similar to the way EN1 modulates mitochondrial signals in adult dopaminergic neurons that regulate cell survival pathways (Alvarez-Fischer, Fuchs et al. 2011). The basal-like breast cancers often result in acquired resistance after a small time course of effective
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Chemotherapy, which necessitates identification of new effective treatments in order to increase patient survival.

A recent study on EN1 showed that an EN iPep interfered with the EN1 TF function and inhibited its oncogenic activity by competing with other proteins, which prevented protein-protein interaction essential for cooperative DNA binding (Beltran, Graves et al. 2014). In addition to that, proteomic analysis demonstrated that EPRS, an inflammatory regulator, binds to the EN1 TF. EPRS is responsible for the translational control of inflammatory proteins and the amino acid stress response (Sundrud, Koralov et al. 2009; Beltran, Graves et al. 2014). Aberrant EPRS function such as mutation and unusual molecular interactions of EPRS with various proteins can interrupt protein synthesis and regulatory networks resulting in various events such as immune response, angiogenesis, transcriptional and translational control (Kim, You et al. 2011). These events are the basis of various diseases including cancer, neuronal pathologies, and autoimmune disorders (Park, Schimmel et al. 2008). So far, the relationship between the EN1 iPep and EPRS remains elusive. This chapter revolves mainly on the implementation of the active EN1 iPep to block the EN1 TF oncogenic activity through the regulation of inflammatory targets using the EPRS pathway.

Here, we demonstrate that inhibiting EN1 TF function using the active EN1 iPep induced apoptosis and significantly reduced cell viability of common human and mouse breast cancer cell lines expressing high levels of EN1 including SUM-149 and T11, which are TNBCs. In addition to the EN1 iPep, these cells were treated with cisplatin, which also resulted in significant reduction of cell viability, consistent with previous studies and confirming the specific sensitivity of TNBCs towards this platinum-based drug. Interestingly, a combination of these two drugs, the EN1 iPep and cisplatin, did not show a synergistic or additive effect. Instead, EN1 iPep suppressed the effect of cisplatin in an unknown manner. The two very different mechanisms and pathways of action involved in the treatment with iPep or cisplatin could have prevented a significant interaction between the drugs from working in synergy or even have additive effects. Further, the uptake of the iPep is thought to be faster and its localization in the area surrounding the nucleus (perinuclear) may also have prevented cisplatin from penetrating into the nucleus and forming cytotoxic adducts with the DNA. In addition to that, the EN1 iPep induced apoptosis within a short period of time compared to other conventional therapies, such as taxol or cisplatin, which clearly take a longer time to act. This may suggest that by the time cisplatin is able to function, the EN1 iPep taken
up cells is already deteriorating, thus the cisplatin effect is concealed. In this respect, a more appropriately timed incubation in a combinational treatment may prove to be effective in synergising the EN1 iPep with cisplatin. As single treatments, the EN1 iPep showed a more potent anti-cancer effect with lower IC\(_{50}\) value in TN-IBC SUM-149 cells compared to cisplatin, suggesting that the EN1 TF specific targeting is more potent in inducing anti-cancer properties in inflammation-based cancer. The EN1 iPep may thus be a novel and important targeted therapy for these patients and warrants further testing in pre-clinical models.

EPRS binding to EN1 TF may lead to aberrant expression such as uncontrolled protein synthesis of inflammatory genes that sustain cell proliferation, eventually leading to tumorigenesis. Proliferation is naturally enhanced during tissue injury or when wound healing is taking place which means the environment surrounding the injury site is enriched in inflammatory cells and growth factors while proliferation stops when the wound healing process is complete (Coussens and Werb 2002). In the basis of tumorigenesis, tumours are categorised as a wound that can’t be healed, hence there is continuous proliferation as well as inflammation. The possible mechanism involved in the EN1 iPep treatment can be explained first through the co-localization of the active iPep with EPRS (Fig. 4A). Mass spectrometry analysis has also detected the binding of EPRS to the EN1 iPep (Beltran, Graves et al. 2013). EN1 protein is expressed in the cytoplasm and nucleus, yet more prevalently in the nucleus. In contrast, the EPRS is more predominant in the cytoplasm although present to some extend also in the nucleus. At 2 hours incubation with the active EN1 iPep, both the iPep and EPRS proteins were highly expressed and co-localization was visible more in the cytoplasm, followed by the nucleus and perinuclear area (Fig 4A). At the 6\(^{th}\) hour of incubation, the co-localization was more dominant in the perinuclear regions and not in the nucleus, in addition to a down-regulation in the total EPRS protein present (Fig. 4A). This unknown interaction between EPRS and EN1 may be the basis of the aberrant development of IBC and other inflammation related disorders and merits further investigation.

In contrast to protein expression, EPRS mRNA expression was not affected by the EN1 iPep; instead, there was a slight increase in expression at each time point (Fig. 5). In parallel to that, mRNA expression of EN1 and an important pro-inflammatory target of EPRS, the VEGFA involved in vascular angiogenesis, were almost as high as EPRS at post 2 hours and 6 hours active EN1 iPep treatment, versus mock and mutant EN1 iPep treated cells. At the 10\(^{th}\) hour, both EN1 and VEGFA expression decreased
significantly although they were still higher than the control cells. This observation may suggest a post-transcriptional regulation control of EPRS, EN1 and VEGFA induced by EN1 iPep-EPRS binding. An important observation that may support this finding was seen in an investigation by (Yao and Fox 2012) whereby VEGFA mRNA and protein showed a linear relationship after a 8-hour treatment with IFN-γ (GAIT system activation), however after 24 hours (GAIT system inhibitory effect takes place), the protein expression was independent of VEGFA mRNA expression. This transcript-specific regulation of VEGFA (Ray et al. 2009) can be explained by the involvement of EPRS as an important component of the interferon (IFN)-γ-activated inhibitor of translation (GAIT) system which leads to GAIT element–specific translation suppression through the binding of GAIT elements in the 3’-UTRs (Brook, Smith et al. 2012; Guo and Schimmel 2013). Based on the similar gene expression pattern of VEGFA and EN1, one may assume that EN1 is a transcript specific target of EPRS. Further study is required to confirm this.

The GAIT system is commonly known to be activated when the AAR pathway is activated, an indication of amino acid starvation or intracellular accumulation of uncharged tRNAs resulting in stress built up in cells. Not much is known about the glutamyl-tRNA synthase, however the Whitman Laboratory has shown that Halofuginone, a plant bioactive compound binds EPRS and inhibited the function of prolyl-tRNA synthase (Keller, Zocco et al. 2012). In regards to this finding, the proline incorporation ability was examined in active EN1 iPep treated SUM-149 and untreated cells (Fig. 4B). Significant reduction in incorporated proline by radioactive labelling in the active EN1 iPep treated cells versus untreated was observed (Fig. 4B). It is believed that the binding of active EN1 iPep to EPRS interferes with EPRS function to load proline to its cognate tRNA leading to intracellular accumulation of uncharged tRNAs.

At the gene level, proline enriched collagen (COL1A1) mRNA expression post 10 hour treatment with the active iPep showing significant down-regulation. Collagen is another pro-inflammatory target of EPRS involved in cell proliferation and malignancy in neoplastic cells (Luparello 2013; Triulzi, Casalini et al. 2013). Collagen is an important component of the extracellular matrix (ECM), which is usually present in the tumour microenvironment along with stromal cells, angiogenic and inflammatory cells. Collagen is often highly expressed in breast cancers, hence it is associated with an increased risk of breast cancer especially in TNBCs (Luparello 2013). In contrast, exogenous proline addition in access (50 µM) to cells treated with the active iPep re-
established cell viability in SUM-149 cells from 70% to 135% viable cells and further enhanced proliferation of mutant iPep treated cells (Fig.4C), while there was no significant changes or effect observed with methionine addition.

These findings indicate that proline specifically competes with the iPep for the active binding site on EPRS as there is a clear binding affinity between the active iPep to the active pocket of EPRS as shown in Fig. 2C. The active pocket of EPRS homes the proline tRNA binding site as well as residues of proline, which is crucial for amino acid metabolism (Son, Lee et al. 2013). However, the active iPep has a stronger affinity towards the EPRS active binding site in which the proline binding site is situated as well. Consequently, proline recognition in the active site, catalysis and delivery to the accepter end of its specific tRNA by EPRS is prevented and blocked. However, proline becomes more competent when it is in excess compared to the active iPep, suggesting that the EN1 iPep specifically inhibits the prolyl- tRNA synthetase of EPRS.

In the typical scenario, the role of an aminoacyl tRNA synthase is programmed to partially shut down the gene switch when there is an unavailability of the particular amino acid causing protein synthesis to slow down in addition to reduced inflammatory cells such as helper T-cells and proliferation, but is activated again when there is sufficient amount of amino acids. Amino acid deprivation most likely induces stress responses in metabolic tissues activating anti-inflammatory downstream targets that disrupt homeostasis (Spriggs, Bushell et al. 2010). This may explain the up-regulation of IL11 (anti-inflammatory) FOXA2 (tumour suppressor) and inhibition of CD69 (pro-inflammatory) genes in SUM-149 post EN1 iPep treatment (Fig. 4). However a long lasting or permanent binding to EPRS active pocket strengthened by hydrophobic interaction through tyrosine (Y) residues on the iPep sequence sustains continuous lack of proline amino acid and its uncharged cognate tRNAs as well as non-functional EPRS. This leads to EPRS being completely switched off and eventually in cellular apoptosis (Cagnol and Chambard 2010; Shore, Papa et al. 2011). Another possibility is that EPRS binding to EN1 iPep prevents it from binding to other components of the GAIT complex, which is followed by EPRS depletion, disrupted amino acid metabolism and finally, induction of cell death. Aberrant function of EPRS in the multisynthetase complex (MSC), which fails to catalise proline could also possibly influence EPRS interaction with other components of the MSC, especially AIMP3. AIMP3 is known to bind to EPRS in the MSC, and the disrupted interaction between EPRS-AIMP3 may also modulate the tumour suppressive activity in the nucleus.
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There seems to be an important link between the EN1 TF and EPRS in relation to amino acid metabolism, inflammation and tumorigenesis, in which tumour cells are protected from degradation. Interfering with the prime function of these two important genes using an EN1 iPep induced tumour cell death in SUM-149 cells representing TN-IBC, potentially by activating the AAR pathway and controlling regulation of inflammatory genes. Therefore, the iPep may be an important therapeutic tool in treating EN1 expression-related cancer and in addition other immuno-compromised diseases in which EN1 manifests a prime function. Further study is needed to prove that the iPep binds transcriptional partners such as EPRS by examining purified EPRS protein binding to the iPep \textit{in vitro}. In addition to that, it will be important to examine whether the changes in expression of the target genes are induced by EPRS-iPep binding and interaction. This aim could be achieved by conducting similar gene expression studies by EPRS knock-down in SUM-149 cells. Lastly, it also would be of great interest to run RNA sequencing to check for global transcriptomal changes caused by the EN1 inhibition.

4.6 Conclusion

EN1 is an important neural-specific TF\textsubscript{HD} and key regulator of the early embryonic developmental stage in the central nervous system (CNS), which is essential in controlling cell identity, growth and differentiation. Overexpression of \textit{EN1} has been linked to the development of breast cancers by promoting survival of tumour cells and resistance towards chemotherapy, especially in TN-IBC. These indicate that EN1 may be an important therapeutic biomarker in cancer. The use of a newly established EN1 interference peptide (iPep) has demonstrated a more potent anti-cancer effect in SUM-149, a TN-IBC representative cell line which had the highest EN1 overexpression, in comparison to cisplatin which is a well-known TNBC chemotherapy drug. The EN1 iPep which blocks the EN1 TF function as an oncogene may provide a novel targeted therapeutic drug specifically for EN1 expressing and inflammation related diseases in addition to cancer.
CHAPTER 5

TARGETING SOX2 VIA INTERFERENCE PEPTIDE (iPep) TECHNOLOGY SENSITISES CANCER STEM CELLS (CSCs) TO CISPLATIN TREATMENT
Targeting SOX2 via interference peptide (iPep) technology sensitises cancer stem cells (CSCs) to cisplatin treatment

5.1 Abstract

SOX2 is an important TF and part of the core transcriptional circuitry regulating pluripotency and self-renewal of ESCs. Overexpression of SOX2 is commonly linked to development of various aggressive cancers such as TNBCs, ovarian and brain cancers, which are enriched in potent CSCs. Cisplatin, a well-known chemotherapeutic agent has shown promising effects in treating TNBCs and ovarian cancers. However, due to the high toxicity caused by cisplatin in normal tissues, there is a great need of effective combinatorial therapies, which with low cisplatin dose still exhibit potent anticancer effects. In Chapter 3 of this thesis, I showed that cisplatin induced differentiation of CSCs in TNBC cells. Here, I investigated cisplatin along with SOX2 inhibition in TNBCs and SOC cells expressing high levels of SOX2. This was achieved using a newly constructed SOX2 iPep in two of the highest SOX2-expressing cell lines, the PA1 SOC and TNBC representing T11 mouse mammary carcinoma cells. Other breast cancer cell lines were also tested together with normal mammary epithelial cells. The iPep was designed and constructed by incorporating a highly conserved sequence, which is found in almost all SOX proteins in the C-terminus and which binds specifically to the DNA interfering with protein-protein interactions between SOX2 and other TFs essential for the SOX2 oncogenic activity. This study investigated if differentiation was induced by inhibiting SOX2 expression and if there was a synergy between cisplatin and the iPep. The SOX2 iPep showed a very clear nuclear localization as well a significant cell viability reduction in a SOX2 expression- and dose-dependent manner, with strongest effect on T11 cells ($IC_{50}=7.35 \, \mu{M}$), followed by PA1 cells ($IC_{50}=16.01 \, \mu{M}$) and MDA-MB-435 cells ($IC_{50}=26.47 \, \mu{M}$). No harm was caused in low/non-SOX2 expressing cancer cells, such as MDA-MB-231, and in normal mammary cells such as HUMEC and MCF-10A, indicating a highly specific function of the iPep in targeting SOX2-expressing cancer cells. Interestingly, unlike the EN1 active iPep (Chapter 4), the SOX2 iPep highly synergized with cisplatin and sensitized T11 and PA1 cells to cisplatin treatment by reducing the $IC_{50}$ values in T11 (13.23 $\mu{M}$ to 3.51 $\mu{M}$) and PA1 (16.25 $\mu{M}$ to 4.39 $\mu{M}$). Flow cytometric analysis and immunofluorescence microscopy demonstrated that both cisplatin and the iPep induced differentiation of cancer cells by significantly down-regulating SOX2 and other stem
cell markers (NANOG, NESTIN, CD133, CD44, SSEA4 and CD49f) as well as reducing cell proliferation between 40-100% while significantly up-regulating differentiation markers (CD24, CK18 and β-tubulin) (68-250%). An enhanced effect was seen in the cisplatin-SOX2 iPep combinational treatment, which almost completely inhibited SOX2 expression and ki67 (proliferation marker). In conclusion, the SOX2 iPep in combination with low cisplatin dose may provide a novel significantly less toxic and effective chemotherapy treatment for patients with breast and ovarian cancers.

5.2 Introduction
SOX2, a family member of the SRY-related HMG box protein 2 (Sox2) is part of the core transcriptional circuitry regulating pluripotency and self-renewal ESCs (Bareiss, Paczulla et al. 2013; Liu, Lin et al. 2013; Wu, Ye et al. 2013). SOX2 is also a key TF which induces pluripotency (Huangfu, Osafune et al. 2008; Kim, Zaehres et al. 2008) in different somatic cell types, and is also normally expressed by neural stem cells promoting and maintaining the stem cell state, while preventing differentiation into neurons (Graham, Khudyakov et al. 2003; Episkopou 2005). In addition to neural stem cells, SOX2 has recently been found to be expressed by other types of adult stem cells, such as those of the breast, particularly during pregnancy and (Hassiotou, Beltran et al. 2012) special populations of stem cells in the bone marrow (Kuroda, Kitada et al. 2010) and endometrium (Park, Seo et al. 2011). Generally SOX2 expression is lost in differentiated cells (Lopez, Valdez-Morales et al. 2013). However, genetic mutations and epigenetic modifications can lead to overexpression of SOX2 in various tissues in a subset of aberrantly proliferating stem-like cells termed CSCs, which are associated with tumorigenesis (Masui, Nakatake et al. 2007; Liu, Lin et al. 2013; Lopez, Valdez-Morales et al. 2013).

Gene amplification and overexpression of SOX2 has been recently identified in breast, ovarian, lung, brain, gastric and Ewing sarcoma cancers (Riggi, Suva et al. 2010; Otsubo, Akiyama et al. 2011; Bareiss, Paczulla et al. 2013; Karamboulas and Ailles 2013; Liu, Lin et al. 2013). SOX2 overexpression is commonly correlated with poor prognosis and low survival in cancer patients (Masui, Nakatake et al. 2007; Liu, Lin et al. 2013; Lopez, Valdez-Morales et al. 2013). In breast carcinomas, SOX2 is mostly detected in a small proportion of tumour-seeding cells, which is an early event in tumour development (Lengerke, Fehm et al. 2011; Leis, Eguiara et al. 2012). In addition, studies have shown that 15-61% of ovarian carcinomas express SOX2 (Zhang,
Chang et al. 2012; Bareiss, Paczulla et al. 2013). The cancer SOX2+ stem-like cells play important roles in maintaining these tumours, especially in high-grade SOC cells (Zhang, Chang et al. 2012). Similarly, presence of CSCs in breast tumours, especially the TNBCs which lack the ER, PR and HER2 receptors (Hombach-Klonisch, Paranjothy et al. 2008; Stolzenburg, Rots et al. 2012; Prabhakaran, Hassiotou et al. 2013) are responsible for the survival of tumour cells. The role of SOX2 as an oncogene in breast cancer has been further supported by studies in our laboratory showing that targeted silencing of SOX2 decreased tumour cell proliferation and colony formation in breast cancer cells (Stolzenburg, Rots et al. 2012). It is interesting that both the TNBCs and SOC are known to carry BRCA1 mutations, and are ER-, highly proliferative aggressive cancers with very poor survival rates and only a 5-year relapse-free period post chemotherapy (Bowtell 2010; Ma, Lai et al. 2010; Schwede, Spentzos et al. 2013). Despite that, both TNBCs and SOC have specific sensitivity towards DNA damaging therapies for example taxol (Ma, Lai et al. 2010; Lee, Shin et al. 2012) and the more potent cisplatin, a cytotoxic based platinum anti-cancer drug (Schewde et al, 2013; Basu and Krishnamurthy 2010; Florea and Busselberg 2011; Pines, Kelstrup et al. 2011).

Cisplatin is known to form Pt-DNA adducts at the 1,2-intrastrand crosslink causing DNA damage, leading to the activation of various signal transduction pathways (Zeidan, Jenkins et al. 2008; Basu and Krishnamurthy 2010; Florea and Busselberg 2011; Wang, Milum et al. 2011). This platinum-based drug has been used comprehensively and effectively in treating breast, ovarian, testicular, cervical, head and neck, and small cell lung cancers (Basu and Krishnamurthy 2010; Florea and Busselberg 2011; Pines, Kelstrup et al. 2011) however the TNBCs and SOC often establish acquired resistance with cisplatin with time (Schwede, Spentzos et al. 2013). It has been shown that various ATP-binding cassette (ABC) transporters, such as the protein encoded by the multidrug resistant gene (MDR), the multidrug resistant protein (MRP), and the breast cancer resistant protein (BCRP1), contribute to chemoresistance in CSCs-enriched cancers (Kondoh, Uchikawa et al. 2004). Thus targeted therapy is crucial for treating these aggressive forms of breast and ovarian cancers. Cisplatin is commonly used in combination with other drugs, such as taxanes, vinca alkaloids, and 5-fluorouracil resulting in additive or synergistic effects (Hollliday and Speirs 2011; Prabhakaran, Hassiotou et al. 2013). In Chapter 3, we showed that cisplatin induced differentiation of TNBC cells which were enriched in CSCs (Prabhakaran, Hassiotou et al. 2013). Based on these highlighted characteristics in TNBCs and SOCs above, there
may be a link between the SOX2 expression, CSC signature and the effects of cisplatin on these tumours.

SOX2 has prevalent roles during development, which are exerted via binding of the SOX2 protein to DNA as well as binding to other TFs and proteins to activate essential molecular and functional pathways (Remenyi, Lins et al. 2003; Kondoh, Uchikawa et al. 2004; Kamachi and Kondoh 2013; Sarkar and Hochedlinger 2013). SOX2 commonly binds and interacts with TFs OCT4, PAX6 and NANOG to initiate self-renewal in normal ESC or oncogenic activity in tumour cells (Kucerova, Dora et al. 2012; Kamachi and Kondoh 2013). It is very difficult to completely diminish TFs and its versatile functions as they possess a special well programmed function, which is presumed to be ‘undruggable’ as transcription is a nuclear event which is not easily accessed by any therapeutic agent (Koehler 2010; Yan and Higgins 2013). Furthermore, it is also known that most essential components involved in transcription lack enzymatic activity that allow chemical intervention (Koehler 2010; Yan and Higgins 2013). A promising method is by blocking the highly competent protein-protein interactions between SOX2 and the other TFs. This can be done using small molecules that target large protein interfaces, which can be done using antisense oligonucleotides, polyamides, RNAi approaches and stable peptides (Nickols, Jacobs et al. 2007; Arora and Ansari 2009).

In this study, we designed an iPep that mimics the highly conserved SOX2 sequence and competes with SOX2 binding partners. SOX2, a member of the SoxB1 family, has short N-terminal sequences followed by the HMG domain and finally the long C-terminal sequences. A number of important protein residues ordering this C-terminal region such as V3, R5, P6, H63, H67, P68, Y71, Y72, R76 and R78, make up the conserved sequence, which binds to the DNA in a sequence-specific manner and is conserved in almost all SOX family members (Remenyi, Lins et al. 2003). The C-terminal sequence consists of the transcriptional activation domain (Kamachi and Kondoh 2013). The PRRK sequence in the activation domain is known as the nuclear localization sequence (NLS) which is aided by the Lysine (K) residue (Zhang, Eden et al. 2012). It has been also shown that K residues are able to mediate penetration of peptide cargos containing hydrophobic residues, such as W and Y (Morris, Deshayes et al. 2008; Zhang, Eden et al. 2012). We demonstrate that this targeted inhibition of protein-protein interactions leads to reduced cell proliferation through the down-regulation of SOX2 and other related stem cell markers, while inducing tumour cell
differentiation by up-regulating differentiation markers. We also show that the SOX2 iPep works synergistically with cisplatin in TNBC cells and SOC cells.

5.3 Materials and Methods

5.3.1 Cell Culture

The cell lines used were (Table 1) were: MCF10A (immortalized human mammary cells), HUMEC (normal human mammary epithelial cells), Mel-2 human embryonic stem cells, mouse embryonic fibroblast (mEF) cells, MCF-7, MDA-MD-231 and MDA-MB-435 (human breast cancer cells), p53 null T11 (mouse mammary carcinoma cells) and PA1 SOC cells (American Type Culture Collection, ATCC). They were cultured in T25 flasks (Corning, Tewksbury MA, USA) at 37°C and 5% CO₂. MCF10A and HUMEC cells were cultured in HuMEC complete medium (Invitrogen, Carlsbad, CA, USA). Mel-2 cells were cultured in Tesr-E8 medium (Stem Cell Technologies) on Matrigel (In Vitro). The remaining cell lines were cultured in basal media supplemented with 10% FBS (Serana, WA Pty Ltd, Bunbury, WA, Australia) and 1% antibiotic-antimycotic (100 U/mL penicillin, 100 µg/0.25 µg/mL streptomycin) (Invitrogen). For MDA-MD-231, MDA-MB-435 and mEF, the basal medium used was Dulbecco’s Modified Eagle Medium (DMEM) (Invitrogen); for T11, RPMI Medium 1640 (RMP1 + Glutmax™1) (Invitrogen); while for MCF-7 and PA1, Minimum Essential Medium (1X) (MEM Alpha + Glutmax™1) (Invitrogen). Supplements such as 1% Sodium bicarbonate (7.5%) (Invitrogen), MEM non-essential amino acid (NEAA) (Invitrogen) and 1% sodium pyruvate (100mM) (Invitrogen) were added to the MCF-7 culture medium. Cells were passaged twice a week at 60-70% cell confluency.

### Table 1. Breast and ovarian related normal and cancer cell lines used.

<table>
<thead>
<tr>
<th>Cell line</th>
<th>Site of origin</th>
<th>Tumour type</th>
<th>Tumour classification</th>
<th>State of differentiation</th>
</tr>
</thead>
<tbody>
<tr>
<td>MDA-MB-231</td>
<td>Pleural effusion</td>
<td>Adenocarcinoma</td>
<td>Claudin-low</td>
<td>poorly differentiated</td>
</tr>
<tr>
<td>MDA-MB-435</td>
<td>Pleural effusion</td>
<td>Infiltrating ductal carcinoma</td>
<td>Basal-like</td>
<td>less differentiated</td>
</tr>
<tr>
<td>MCF10A</td>
<td>Normal breast</td>
<td>Fibrocystic</td>
<td>Non-tumorigenic</td>
<td>Highly differentiated</td>
</tr>
<tr>
<td>PA-1</td>
<td>Ovary: Ascites</td>
<td>Teratocarcinoma</td>
<td>P53 null mammary</td>
<td>Poorly differentiated</td>
</tr>
<tr>
<td>T11</td>
<td>mouse breast</td>
<td></td>
<td>claudin-low</td>
<td>Poorly differentiated</td>
</tr>
</tbody>
</table>
5.3.2 Determination of Cell Viability

Cell cultures of 60-70% confluency were used for experiments. A 100-µL of $3 \times 10^3$ cells supplemented with 5% FBS and 1% antibiotic-antimycotic per well were seeded in flat-bottom 96-well plates (Sarstedt, Newton, USA). A lower FBS percentage was used for all the experiments in order to minimise interference between the iPep and FBS, while still providing sufficient growth needs for the cells. After 24 hours, SOX2 iPep (C516) (ChinaPeptides Co., Ltd., China) and cisplatin (Sigma-Aldrich, St Louis, MO, USA) were directly added into cells respectively at different concentrations each (2.5, 5, 10, 15, 20, 25 and 50 µM). These concentrations were chosen according to the range of cisplatin concentrations used in clinical treatments. In the iPep-cisplatin combination treatment, a fixed concentration of 5 µM SOX2 iPep was added two hours prior to various cisplatin concentrations (2.5, 5, 10, 15, 20, 25 and 50 µM). The iPep was added first to the cells as a preventive step to avoid the risk of peptide digestion by DMSO, in which cisplatin is dissolved. The 5 µM SOX2 iPep concentration was chosen because it reduced cell viability significantly in the single treatment and the aim of the combinational treatment was to identify an effective low cisplatin dose that may induce a significant anti-cancer effect in synergy with the iPep. Cells were incubated with each treatment for a total of 12 hours before cell viability was assessed by MTS colorimetric assay, using Cell Titer 96® Aqueous (Promega, Madison, USA), according to the manufacturer’s instructions. The MTS substrate was added (10-µL in 100-µL medium) and incubated for 4 hours. Finally the MTS activity was assayed at 490 nm using a plate reader (Labsystems Multiskan RC). The higher the number of viable cells, the higher is the purple color intensity. The IC$_{50}$ of each drug treatment were calculated using the GraphPad Prism 6 software. This was done in quadruplicate in three independent experiments.

5.3.3 Immunofluorescence Microscopy and iPep Internalisation

Cells ($3 \times 10^4$ cells) were grown on glass coverslips in 24-well plates (Sarstedt) for 24 hours. In iPep internalization microscopy, fluorescence tagged (P870) SOX2 iPep synthesized by the UNC High-Throughput Peptide Synthesis and Array Facility at UNC (Chapel Hill, North Carolina, USA) were used. PA1 and T11 cells were incubated with the iPep for 2 hours in serum-free medium to eliminate interference between the iPep and FBS, followed by fixation with 1% PFA in PBS/2% sucrose, permeabilised with
0.1% Triton X-100 in PBS for 30 minutes, DAPI (Roche, 1:100) nuclear staining for 10 minutes and finally coverslips are transferred onto glass slides. Cells on the coverslips are washed (2x) with PBS at each step. In contrast, detection of protein expression was done by treating PA1 cells with 15 µM SOX2 iPep, 15 µM cisplatin as well as a combination (5 µM SOX2 iPep+5 µM cisplatin) respectively and incubated for 12 hours. A non-fluorescence tagged iPep (Ca-516) (ChinaPeptides Co., Ltd., China) was used. These concentrations were chosen based on the IC₅₀ values for each treatment, hence were used in all experiments. The PA1 cells were incubated with each treatment 12 hours in similar conditions (5% FBS+1%1% antibiotic-antimycotic) as mentioned previously in section 5.3.2.

Cells were then fixed in 1% paraformaldehyde (PFA) in 2% sucrose in PBS, permeabilised with 0.1% Triton X-100 in PBS for 30 minutes, incubated overnight with primary antibodies (Suppl. Table 1), and then incubated for 4 hours with secondary antibodies (Suppl. Table 1) and DAPI (Roche, 1:100) for nuclear staining. Appropriate negative controls (secondary antibody only) were used. Coverslips with cells were then transferred onto glass slides with fluorescence mounting medium (Dako) and sealed with colourless nail polish. Cells were imaged using an upright Nikon Eclipse 90i microscope.

5.3.4 Determination of Caspase-3 processing

PA1 ovarian cancer cell cultures of 60-70% confluency were passaged by trypsinisation. A day later, the cells were treated with 15 µM SOX2 iPep and 10 µM taxol respectively and incubated for 12 hours. Taxol is a common DNA-damaging drug used in treating TNBCs as well as ovarian cancers, and it induces apoptosis in cancer cells, with 10 µM being the IC₅₀ concentration that induces apoptosis in PA1 cells (Ma, Lai et al. 2010; Lee, Shin et al. 2012). Hence, taxol was used as a positive control while untreated PA1 cells as negative control in the assay. Cells were then gently scraped and centrifuged at 145 g for 5 minutes. Protein extraction was done using RIPA buffer in which the final supernatant (protein) collected was further used in protein quantification (Bradford assay), followed by apoptosis detection with a Human Caspase-3 (active) colorimetric assay (Invitrogen ELISA Kit, Catalog# KHO1091, Carlsbad, CA, USA), according to the manufacturer’s instructions. Briefly, appropriate standards and cell extracts were incubated at room temperature for 2 hours in the caspase-3 antibody-coated wells. Next, the wells were incubated with detection antibody for an hour,
followed by HRP anti-rabbit antibody incubation for 30 minutes. This step was then continued with further 30 minutes incubation with stabilized chromogen and finally a stop solution was added. Each solution in samples was aspirated and wells were washed prior to each step. Readings were taken using a plate reader at 450 nm. The higher the active caspase-3 activity in samples, the higher is the blue colour intensity.

5.3.5 Protein Immunodetection by Flow Cytometry
PA1 ovarian cancer cell cultures of 60-70% confluency were passaged by trypsinisation. A day later, the cells were treated with 15 µM SOX2 iPep (Ca-516), 15 µM cisplatin and the combination (5 µM SOX2 iPep + 5 µM cisplatin) respectively and incubated for 12 hours. Cells were then gently scraped and centrifuged at 145 g for 5 minutes. Cells were fixed in 1% PFA in 2% sucrose in PBS for 20 minutes at room temperature, and incubated with primary antibodies (Suppl. Table 1) for 1 hour at 4°C, followed by incubation with secondary antibodies (Suppl. Table 1) for 30 minutes at 4°C. All intracellular marker antibodies were prepared in permeabilisation solution (0.05% Tween-20 in PBS), whilst surface marker antibodies were prepared in 7% FBS in PBS. Appropriate negative controls (secondary antibody only) were also used. Cell pellets were washed at least twice after each step. Sample washing was done using 0.05% Tween-20 in PBS and 7% FBS in PBS for intracellular and surface markers, respectively. Data acquisition was done with a FACS Calibur Flow Cytometer (Becton Dickinson, New Jersey, USA), and 10,000 events were collected and analysed per sample. FlowJo was used for data analysis. Expression levels were analysed as the standardised difference in the Mean Fluorescence Intensity (MFI) between the control and the test.

5.3.6 mRNA Quantification by Taqman qRT-PCR
 Cells were seeded and treated using similar protocols as found in Chapter 3 (Heading 3.3.5) and Chapter 4 (Heading 4.3.7). Total cellular RNA was extracted using RNAzol®RT (Molecular Research Center, Inc). RNA quantity and quality were assessed with NanoDrop 1000 (NanoDrop, Wilmington, DE). For each sample, 5 µg RNA was treated with RQ1 RNase-Free DNase (Promega, Madison, USA) and was reverse transcribed using MMLV (Promega) by incubating at 25°C (10 minutes), 55°C (50 minutes) and 70°C (15 minutes) using PTC-100 ™ Programmable Thermal Controller (MJ Research Inc.). The RT Reaction Clean-Up MoBio kit (MoBio Lab Inc., CA, USA)
was used for cDNA clean up. The qRT-PCR reaction was performed with TaqMan Fast Universal Master Mix (Applied Biosystems, Carlsbad, CA). A 20 µL reaction mixture per well was used, which consisted of 10 µL Master Mix, 1 µL of primer for each gene (Suppl. Table 3), 3.5 µL of RNAse free H2O and 150 ng of cDNA to detect the relative abundance of transcripts. The conditions for all qRT-PCR reactions were as follows: 20 minutes at 95°C (holding stage) followed by 3 seconds at 95°C and 30 seconds at 60°C cycling stage) for 40 cycles. GAPDH (Table 2) was used as the internal positive control and non-template sample as the negative control. A comparative \( \Delta \Delta C_T \) was used to analyse the SOX2 (Table 2) mRNA expression.

TABLE 2. TaqMan primers used for RT-PCR

<table>
<thead>
<tr>
<th>Gene</th>
<th>Specification</th>
<th>Catalogue No.</th>
</tr>
</thead>
<tbody>
<tr>
<td>GAPDH</td>
<td>Hu GAPDH (20X) FAM MGB</td>
<td>4333764-1207037</td>
</tr>
<tr>
<td>GAPDH</td>
<td>Mouse GAPDH (20X) FAM MBG</td>
<td>4352932-0802019</td>
</tr>
<tr>
<td>SOX2</td>
<td>Mm00488369_s1</td>
<td>1230199 D11</td>
</tr>
<tr>
<td>SOX2</td>
<td>Mm00488369_s1</td>
<td>957418 E1</td>
</tr>
</tbody>
</table>

5.3.7 Statistical Analysis

Statistical analysis and graphical exploration of the data were done in Microsoft Excel. The Student’s paired \( t \)-test with a two-tailed distribution was used to compare each treatment to its untreated/vehicle cells, respectively. The results are presented as mean ± SEM (MTS, flow cytometry and qRT-PCR), as indicated in the corresponding figure legends. The significance is shown as follows: \( \ast p \leq 0.05; \ast \ast p \leq 0.01 \ast \ast \ast p < 0.0001 \).
5.4 Results

5.4.1 SOX2 interfering peptide (iPep) structure, cellular uptake and nuclear localization

To inhibit the action of SOX2 as a TF in cancer cells possessing stem cell properties, we engineered synthetic iPep against HMG TF, comprising the SOX family. The ability of SOX2 to dimerise onto DNA in distinct conformational arrangements as a result of binding and highly conserved surface interaction \textit{in vitro} with other TFs such as OCT4 and PAX6 has been previously shown to be crucial for transcriptional regulation (Remenyi, Lins et al. 2003; Karnavas, Mandalos et al. 2013). We reasoned that the delivery of an interfering peptide, which mimics these highly conserved protein-protein interactions, would compete with the SOX2 binding partners (OCT4 and PAX6) and inhibit the TF oncogenic activity (Fig. 1A). Figure 1B shows the designed SOX2-iPep sequence consisting of 24 amino acids, which mimics the sequence of the HMG domain, including the consensus sequence.

In order to confirm that the peptide is taken up into cells, internalization studies were performed. The iPep was coupled to a C-terminal fluorescein molecule and delivered into T11 and PA1 cells, which are known to express high levels of SOX2 (Bareiss, Paczulla et al. 2013). Cells were imaged after two hours using an upright Nikon Eclipse 90i microscope. Real-time imaging over a 2-hour period was also conducted using a Nikon inverted microscope. The SOX2 iPep was directly taken up into the nucleus with specific nuclear localization within 2 hours (Figure 1B-C; Suppl. Video).
Figure 1. SOX2 interfering peptide (iPep) structure, cellular uptake and localization. (A) SOX2 iPep structure and (B) sequence. Figures A and B were designed and constructed by Pilar Blancafort. (C and D) SOX2 iPep cellular uptake in T11 and PA1 cancer cells. The SOX2 iPep is directly taken up into specific locations in the nucleus. Cells were incubated with 15 µM SOX2 iPep for 2 hours. Scale bar: 50µm.

5.4.2 SOX2 is overexpressed in breast and ovarian cancer cells that are affected by the SOX2 iPep

Successful iPep internalization and uptake in T11 and PA1 lead us to also investigate the effect of the iPep on other cancer cells. Hence, gene expression of SOX2 was examined in a panel of various cell lines, which express SOX2 at different levels. These included HUMEC and MCF10A (normal human mammary cells), human embryonic stem cells (hESC), MCF7, MDA-MB-231 and MDA-MB-435s (human breast cancer cells), PA1 (SOC) as well as mouse embryonic fibroblast (mEF) and T11 mouse mammary carcinoma cells. MCF7 represented the luminal breast cancer subtype, while MDA-MB-231, MDA-MB-435 and T11 are TNBC cells. The SOX2 mRNA expression study as seen in Fig. 2A clearly shows that PA1 cells overexpress SOX2 compared to normal ESCs, followed by MCF-7, MDA-MB-435, MDA-MB-231, MCF10A and HUMEC.
Figure 2. **SOX2 expression in breast and ovarian cancer cells.** (A) Expression of SOX2 across various human (HUMEC, MCF10A, MDA-MB-231, MDA-MB-435s and PA1) and mouse (T11) cancer cell lines. Figure A was produced by Jessie O’Mahony. (B) Effect of SOX2 iPep in some of these cells at increasing concentration (0, 2.5, 5, 10, 15, 20, 25 and 50 µM) on cell viability which was determined by MTS assay. The sigmoid graphs show the IC\textsubscript{50} value of the SOX2 iPep treatment.

The mouse line T11 also expresses very high levels of SOX2. Next, the SOX2-iPep inhibitory effect was tested in most of these cell lines including MCF10A, MDA-MB-231, MDA-MB-435, PA1 and T11. The iPep showed significant inhibition of cell viability. SOX2 iPep treated MDA-MB-435 cells demonstrated an IC\textsubscript{50} value of 26.47 µM, PA1 cells of IC\textsubscript{50}=16.01 µM, and T11 cells of IC\textsubscript{50}=7.35 µM. In contrast, MDA-MB-231 and MCF10A cells, which have very low SOX2 expression, were unaffected even at 50 µM SOX2 iPep (Fig 2B). These experiments indicate that the reduction in cell viability induced by the SOX2 iPep was specific and dependent on the initial
expression of SOX2, with the higher expressers incurring the greatest effect. A higher iPep dose may be needed for a significant effect of the iPep in the cancer cells expressing lower SOX2 levels.

### 5.4.3 SOX2 iPep and cisplatin reduced cell viability synergistically in breast and ovarian cancer cells

Based on the SOX2 expression and the sensitivity of SOX2-expressing cancer cells to the SOX2 iPep (Fig 2A-B), T11 and PA1 cell lines, which represent highly invasive cancers with very high SOX2 levels, were further studied for the effect of the iPep alone and in combination with cisplatin. Both T11 and PA1 cells are commonly known to be very sensitive to cisplatin treatment. Both drugs showed dose-dependent effects in T11 and PA1 cancer cells. A small but significant decline in cell viability was observed beginning from 2.5 µM SOX2 iPep (15%, \( p<0.0005 \)) and cisplatin (11%, \( p<0.005 \)) in treated PA1 cells, achieving a 50% reduction (IC\(_{50}\)) at 16.01 µM and 16.25 µM, followed by 88% \( (p<0.0001) \) and 81% \( (p<0.0001) \) reduction at 50 µM, respectively (Fig. 3B, Table 3). In T11 cells, a similar but higher sensitivity towards both treatments was observed. At 2.5 µM of each SOX2 iPep and cisplatin, T11 cells showed 20% \( (p<0.0001) \) and 13% \( (p<0.005) \) (Fig. 3A, Table 3) reduction in cell viability respectively. At 5 µM, T11 cells showed 30% \( (p<0.0001) \) and 18% \( (p<0.005) \) reduction with the iPep and cisplatin respectively (Fig. 3A, Table 2) compared to 20% \( (p<0.0001) \) and 13% (0.005) in PA1 cells (Fig. 3B, Table 3) at the same dose. A 50% reduction in cell viability was achieved with the SOX2 iPep at 7.35 µM and 13.23 µM for cisplatin, followed by a 90% \( (p<0.0001) \) and 79% \( (p<0.0001) \) reduction at 50 µM, respectively in T11 cells (Fig. 3A, Table 3).

**Table 3:** Summary of cell viability reduction in PA1 and T11 cells by the different treatments tested.

<table>
<thead>
<tr>
<th></th>
<th>IC(_{50}) (µM)</th>
<th>2.5 µM</th>
<th>5 µM</th>
<th>50 µM</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>PA1</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>SOX2 iPep</td>
<td>16.01</td>
<td>15% ( p&lt;0.0005 )</td>
<td>20% ( p&lt;0.0001 )</td>
<td>88% ( p&lt;0.0001 )</td>
</tr>
<tr>
<td>Cisplatin</td>
<td>16.25</td>
<td>11% ( p&lt;0.005 )</td>
<td>13% ( p&lt;0.005 )</td>
<td>81% ( p&lt;0.0001 )</td>
</tr>
<tr>
<td><strong>T11</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>SOX2 iPep</td>
<td>7.35</td>
<td>20% ( p&lt;0.005 )</td>
<td>30% ( p&lt;0.0001 )</td>
<td>90% ( p&lt;0.0001 )</td>
</tr>
<tr>
<td>Cisplatin</td>
<td>13.23</td>
<td>13% ( p&lt;0.005 )</td>
<td>18% ( p&lt;0.005 )</td>
<td>79% ( p&lt;0.0001 )</td>
</tr>
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Figure 3. SOX2 iPep and cisplatin reduce cell viability in breast and ovarian cancer cells. (A and B) Effect of SOX2 iPep, cisplatin and the combination (SOX2 iPep + cisplatin) in T11 mouse mammary carcinoma and PA1 ovarian carcinoma cells, respectively, at increasing concentrations (0, 2.5, 5, 10, 15, 20, 25 and 50 µM) on cell viability, which was determined by MTS assay. The sigmoid graphs show the IC$_{50}$ value of each treatment.

In addition to SOX2 iPep and cisplatin as single therapeutic agents, a combination of both drugs was tested in PA1 and T11 cells. A fixed concentration of 5 µM SOX2 iPep and various cisplatin concentrations (0-50 µM) were administered to the cells. Interestingly, a synergistic effect was observed between the two drugs, which significantly reduced the IC$_{50}$ value versus single drug treatment in both PA1 and T11 cells. The IC$_{50}$ value of the combination treatment in T11 cells was 3.51 µM and 4.39 µM in PA1 cells. The fact that about 50% of PA1 cancer cells still survived with quite high concentration (16 µM) of SOX2 iPep and cisplatin treatment may suggest that these drugs do not directly induce apoptosis, but rather interfere with cellular functions that indirectly cause cell apoptosis.

5.4.4 SOX2 iPep induces cancer cell differentiation

In order to determine the mechanism of action of the SOX2 iPep alone and in combination with cisplatin, the human ovarian cancer cell line PA1 was subjected to an active Caspase-3 ELISA assay, with taxol-treated PA1 cells as the positive control. PA1 cells treated with 15 µM SOX2 iPep showed no change in the caspase-3 activity, while a significant increase ($p<0.05$) was observed in 10 µM taxol treated cells. This suggested that the iPep induced cellular differentiation, instead of apoptosis (Fig. 4A), which was further examined.
We first tested proliferation capacity in PA1 cells. Expression of ki67, a proliferation marker was examined in SOX2 iPep treated cells, and also in cells treated with cisplatin, and the combination SOX2 iPep-cisplatin. Fig. 4B shows that the proliferating capacity was high in untreated cells, but it was down-regulated upon SOX2 iPep or cisplatin single treatments ($p<0.0001$; 85% and $p<0.0001$; 69.8% respectively). Furthermore, the combined SOX2 iPep-cisplatin treatment resulted in almost a complete inhibition of ki67 expression ($p<0.0001$; 95.4%) (Fig. 4B-D; Suppl. Table 3). The fact that the treated cancer cells were still viable despite a limited or no proliferative capacity suggested that the SOX2 iPep and cisplatin induced differentiation of SOX2-expressing CSCs.

The effect of SOX2 iPep, cisplatin and the combined SOX2 iPep-cisplatin treatments on expression of a variety of key markers, such as CD133, CD44, CD24 SSEA4, CD49f, SOX2, NANOG, OCT4, NESTIN, SMA, CK18 and β-tubulin, was examined using flow cytometry. A cellular hierarchy was observed in the untreated PA1
cells (Fig. 5A and B), which expressed various stem cell markers at different levels. NESTIN showed the highest expression, followed by CD133, SOX2, NANOG, CD44, SSEA4, CD49f, OCT4 and CD24 (Fig. 5A and B). In addition, PA1 expressed differentiation markers, mainly CK18, an epithelial marker, and β-tubulin, a microtubule marker. SMA, a myoepithelial marker was not expressed or had negligible expression.

Treatment with 15 µM SOX2 iPep, 15 µM cisplatin and a combination 5 µM (SOX2 iPep+cisplatin) respectively shifted the cellular hierarchy of PA1 cells, causing distinct changes in cell phenotype and protein expression (Fig. 5 and 6; Suppl. Table 3). The stem cell markers NESTIN, CD133, SOX2, NANOG, CD44, SSEA4 and CD49f were significantly down-regulated in 15 µM SOX2 iPep treated PA1 cells (p<0.0001), while SSEA4 (p=0.032) and CD49f (p=0.026) expression decreased, but not to the same extent. Similarly, 15 µM cisplatin and the combination also showed significant reduction in NESTIN (p<0.0001), CD133 (p<0.0001 and p<0.0005, respectively), SOX2 (p<0.0001), NANOG (p<0.0005 and p<0.0001, respectively), CD44 (p<0.0005 and p<0.0001, respectively), SSEA4 (p<0.05 and p<0.0001, respectively), CD49f (p<0.05 and p<0.0005, respectively) as well as OCT4 expression (p=0.022 and p=0.045, respectively) (Fig. 5B; Suppl. Table 3).

In addition to down-regulation of stem cell markers, all three treatments induced significant up-regulation of differentiation markers, including CK18 (p=0.018, p=0.011 and p<0.0001, respectively) and β-tubulin (p<0.0001, p<0.005 and p<0.0001, respectively) (Fig. 5B and 6; Suppl. Table 3). CD24, which is expressed at higher levels by differentiated mammary cells, significantly increased as well (p<0.0001, p<0.005 and p<0.0001, respectively), while only a marginal change in SMA expression was observed upon each treatment (p=0.59, p=0.27 and p=0.31) (Fig. 5B; Suppl. Table 3).

Immunofluorescence imaging confirmed the increased expression of CK18 and β-tubulin along with reduced SOX2 expression upon 15 µM SOX2 iPep, 15 µM cisplatin and (5 µM SOX2 iPep + 5 µM cisplatin) treatments (Fig. 6). These results provided evidence of differentiation induction in the examined ovarian cancer cells. Upon each treatment, the less differentiated PA1 cells displayed a more differentiated phenotype respectively (Fig. 6), with SOX2 iPep treated cells possessing neuron-like morphology, cisplatin treated cells looking enlarged and elongated (Fig. 6), and the SOX2 iPep-cisplatin treated cells appearing contracted, elongated and smaller compared to the untreated/vehicle PA1 cells (Fig. 6).
5.5 Discussion
Recent efforts are focusing on the development of potent treatments for commonly affected cancers and are often difficult to treat with conventional therapies. The TNBCs and SOCs are responsible for the majority of cancer-related deaths among women worldwide. Thus, development of new targeted therapies is needed for these cancers as they are often resistant to conventional treatments. A clinicopathological study by Kutomi and co-workers in Japan revealed 68.5% TNBC patient samples expressed SOX2 (Kutomi, Ohmura et al. 2012) while Zhang and co-workers in the United States showed that 80% of SOC samples analysed among ovarian carcinoma patients had SOX2 overexpression (Zhang, Chang et al. 2012). SOX2 expression is often positively correlated with less differentiated phenotype (Kutomi, Ohmura et al. 2012; Sun, Zhang et al. 2012). In addition, high SOX2 expression in these cancers can be related to CSC enrichment that is responsible for tumour progression, metastasis and recurrence (Bareiss, Paczulla et al. 2013). It is crucial to develop therapies, which specifically target the CSC population and most importantly the genetic networks responsible for the maintenance and treatment resistance of CSCs. As SOX2 TF has a role in cell self-renewal and maintenance of embryonic stem cell state, targeting SOX2 may provide novel treatment options for these cancers. Given the resistance of CSCs to chemotherapy, successful treatments must first induce CSC differentiation to make them more susceptible to the killing effects of other anti-cancer drugs.

Here, we demonstrate that inhibiting SOX2 TF function induced differentiation of common cell lines overexpressing or highly expressing SOX2 including TNBCs and SOC, which are among the most aggressive and highly populated by CSCs. This effect of SOX2 inhibition/knockdown is demonstrated by using a novel SOX2 iPep as well as cisplatin via down-regulation of SOX2 and other stem cell markers such as NANOG, CD133, CD44, CD49f and SSEA4, and subsequent cytotoxicity resulting in marked reduction in cell viability and proliferation. These findings propose a novel approach in drugging the undruggable TFs in tumours, and provide insight into the mechanisms that first differentiate and then kill cancer cells. We designed an iPep, which comprised a sequence known to block protein-protein interactions between SOX2 and other TFs. We designed Ca-516 iPep.
Figure 5. SOX2 iPep and cisplatin shift ovarian cancer cells towards a more differentiated phenotype. Flow cytometric quantification of CD49f, SSEA4, CD44, CD24, NESTIN, SOX2, NANO6, OCT4, SMA, CK18 and β-tubulin protein expression showed various levels in PA1 ovarian carcinoma cell line. (A) Flow cytometry histograms of expression of the above markers in PA1 (grey dashed line: unstained control; black line: FITC-stained untreated cells/vehicle; red line: FITC-stained 15 µM SOX2 iPep-treated cells; blue line: FITC-stained 15 µM cisplatin-treated cells; * line: FITC-stained 5 µM SOX2 iPep + 5 µM cisplatin-treated cells). (B) The bar charts show quantification of the level of expression based on the mean fluorescence intensity (MFI) standardised difference of SOX2 iPep, cisplatin, SOX2 iPep + cisplatin-treated (12 hours) and untreated PA1 cells. Bars are presented as mean fluorescence intensity (MFI) standardised difference±SEM (n=3). *p≤0.05, **p≤0.01***p<0.0001
Figure 6. SOX2 iPep and cisplatin influence expression of SOX2 and differentiation markers in PA1 cells. Differentiation markers (SMA, CK18 and β-tubulin) and SOX2 were checked for expression. CK18 and β-tubulin were up-regulated while Sox2 expression was down-regulated upon treatment with 15 µM cisplatin and SOX2 iPep respectively, as well as combination (5 µM SOX2 iPep + 5 µM cisplatin)-treated PA1 cells. The mouse anti-β-tubulin was used to stain microtubules (green), whilst DAPI nuclear stain was used to stain the nucleus (blue). Scale bars: 50 µm using the structural information from the SOX2-OCT4 (Masui, Nakatake et al. 2007) and SOX2-PAX6 interactions (Remenyi, Lins et al. 2003). The most crucial step subsequent to iPep generation was to examine the iPep cellular internalization and
localization. The cellular uptake is important in order to confirm that the inhibitory effects were the result of the SOX2-targeting iPep. A real time video taken using fluorescence tagged iPep in T11 cells showed clear and instant uptake into the cells (Suppl. Video). The iPep had nuclear localization as the nuclei were lit up within 2 hours indicating potential DNA binding capacity, which also correlates with the target specific site of SOX2 where its role as a TF is active.

Next, a gene expression study was done to screen expression of SOX2 in various cell lines including normal and cancer cells. As seen in Fig. 2, PA1, a SOC cell line clearly expresses abundant levels of SOX2, even more than normal ESC, which is in agreement with other studies conducted on SOCs (Zhang, Chang et al. 2012; Bareiss, Paczulla et al. 2013). Based on this gene expression, some of the cell lines tested for SOX2 expression (Fig. 2A) were also subjected to cell viability assays, which revealed differential potential of the iPep in a strict correlation with the endogenous levels of SOX2 (Fig. 2B) (Morris, Deshayes et al. 2008). We showed that the active iPep comprising the wt SOX2 highly conserved sequence selectively targeted cancer cells expressing high SOX2 levels, potentially by interfering or competing with SOX2 partners in the cancer cells, while leaving the normal cells unharmed which makes the peptide a viable specific anti-cancer therapeutic option. PA1 cells (SOC) and T11 cells (TNBC) demonstrated the highest SOX2 expression and also sensitivity towards the iPep, with an IC$_{50}$ of 7.35 µM and 16.01 µM, respectively (Fig. 3). The lower the SOX2 expression was, the higher iPep dose was required for significant effects. However, it is unclear as to why the mouse cancer cell line is more sensitive to the SOX2 iPep compared to the human cancer cell lines. On the other hand, cisplatin treatment in these two cell lines also showed significant cytotoxicity, being more potent in T11 (IC$_{50}$ = 13.23 µM) followed by PA1 cells (IC$_{50}$ = 16.25 µM) (Fig. 3). Generally, 10-20 µM cisplatin are doses most commonly reached in tissues in clinical treatments. However, the appropriate doses for SOX2 iPep are yet to be identified in the clinical setting. Another important observation was that there were about 10-20% of cancer cells still viable even at higher than 20 µM concentration of SOX2 iPep and cisplatin. It will be important to identify the nature of the surviving cells and whether they had CSC features or were more differentiated cells. Our data suggest that these cells don’t undergo apoptosis but a different mechanism altogether. At higher than 20 µM doses, cisplatin and the iPep probably kill the tumour cells by interfering with the cell structure and function at the DNA level, as has been shown previously with cisplatin (Basu and
Krishnamurthy 2010; Prabhakaran, Hassiotou et al. 2013). At the same time, both SOX2 iPep and cisplatin appeared to regulate gene expression, and therefore induce cellular differentiation, both at the mRNA and protein levels. However, the specificity of both drugs in relation to SOX2 expression respectively varies. This can be explained by the highly specific function of the iPep only when there is abundant SOX2 expression in tumour cells, while cisplatin reduced the cell viability both in SOX2-expressing and SOX2 non-expressing TNBCs, such as MDA-MB-231 and BT-549 cells (Prabhakaran, Hassiotou et al. 2013).

In addition to the effects of the SOX2 iPep and cisplatin as a single therapeutic agent, a more promising significant anti-cancer effect was seen by combining the two treatments. A synergism is observed in the SOX2 iPep-cisplatin combinatorial treatment, which increased sensitivity of cisplatin by reducing the IC$_{50}$ values in both T11 (3.51 µM) and PA1 cells (4.39 µM) (Fig. 3). A fixed iPep concentration (5 µM) was added to the cells 2 hours prior to various cisplatin doses to allow iPep internalization without disruption. Cisplatin at 10 µM is considered sub-lethal, while more than 20 µM is lethal and can cause severe nephrotoxicity in patients (Arany and Safirstein 2003; Ali and Al Moundhri 2006; El-Ghiaty, Ibrahim et al. 2014). Cisplatin dose is usually reduced by 20% or more after a few treatments in some patients due to toxicity (Ang, Harris et al. 2005; Staudacher, Cottu et al. 2011). Hence it is highly recommended to reduce the dosage as much as possible but retaining its potency, which can be achieved through combinatorial treatment (Florea and Busselberg 2011; Holliday and Speirs 2011; van Jaarsveld, Helleman et al. 2013; El-Ghiaty, Ibrahim et al. 2014). In this respect, our results showing sensitisation of cisplatin-treated cells by the SOX2 iPep can have significant clinical applications. It is important to take into consideration that response to these agents may also depend on the properties and cellular hierarchy manifested by each tumour given that tumour cells with high CSC content and less differentiated properties are sensitive to cisplatin (Wang, Milum et al. 2011; Prabhakaran, Hassiotou et al. 2013; van Jaarsveld, Helleman et al. 2013).

Flow cytometric analysis of protein expression confirmed a less differentiated phenotype in human SOC PA1 cells. The stem cell markers NESTIN, SOX2, NANOG, CD44 and CD133 were highly expressed, while SSEA4 and CD49f where expressed at low levels (Fig. 5; Suppl. Table 5). High SOX2 protein expression in SOC is often correlated with histopathologically and clinically aggressive disease and worst survival rate among all other ovarian cancers (Kurman and Shih Ie 2010; Lara, Wang et al. 2012;
Bareiss, Paczulla et al. 2013). High expression of these markers along with ki67, a proliferation marker may explain the high proliferation capacity of PA1. CD24, CD44, CD133, CD49f and SSEA4 surface markers have been commonly used for the detection of CSCs in solid tumours, including human breast, brain, colon and ovarian cancer (Zhou, Zhang et al. 2009), as well as for categorising ovarian and breast cancer molecular subtypes (Hergueta-Redondo, Palacios et al. 2008; Nakshatri, Srour et al. 2009; Stagg and Pommey 2009). Initially, untreated PA1 cells had negligible expression of CD24 and SMA, some expression of CK18, and medium β-tubulin expression (differentiation markers) (Fig. 5; Suppl. Table 3), indicating epithelial cell origin, which is however aberrantly down-regulated in ovarian tumour cells (Lara, Wang et al. 2012; Bareiss, Paczulla et al. 2013). SOX2 i Pep treatment shifted this cellular hierarchy towards more differentiated cells by selectively targeting SOX2 and inhibiting its interaction with their binding partners/TFs crucial in enhancing the oncogenic function.

The down-regulation of SOX2 resulted in down-regulation of SOX2 TF partners such as NANOG and OCT4, which are also involved in ES cell maintenance and fate.

The high expression of both SOX2 and NANOG prior to i Pep treatment and significant down-regulation post treatment may suggest that the i Pep interfered and prevented binding of SOX2 and NANOG. The inhibition of SOX2 and the interacting proteins subsequently down-regulated the other stem cell markers as well which in turn increased CK18, CD24 and β-tubulin as an indication of differentiation. This induction of differentiation was also confirmed by a Caspase-3 ELISA assay, which showed that taxol-treated PA1 cells underwent apoptosis (increase in active caspase-3 protein expression), while SOX2 i Pep treated PA1 cells showed no change in active caspase-3 release. Taxol was used as a positive control in this assay as taxol treatment is known to induce apoptosis in PA1 cells (Ma et al, 2010; Lee et al, 2012).

The blocking of protein-protein interaction initiated by the i Pep significantly down-regulated SOX2 (84.7%) expression, but also the binding partner/TF such as NANOG (71.8%), along with NESTIN (neural specific, 33.6%) and other stem cell markers CD44 (64.3%) and CD133 (88%) as well as ki67 (85%) (Fig. 5; Suppl. Table 3). In addition, OCT4 (18.4%) another important TF and binding partner of SOX2, CD49f (15.7%) and SSEA4 (84.7%) were also significantly down-regulated although the initial expression was very low (Fig. 5; Suppl. Table 3). In contrast to the stem cell markers down-regulation, an up-regulation of differentiation markers by 60-180% were observed (Fig. 2; Suppl. Table 3). High NESTIN, SOX2, NANOG, CD44, CD133
CD49f and SSEA4 expression has been associated with low levels of tumour differentiation and reduced survival in ovarian and breast cancer patients (Zeidan, Jenkins et al. 2008; Stagg and Pommey 2009; Meyer, Fleming et al. 2010; Sanges and Cosma 2010).

The differentiation marker CK18 is highly expressed in the normal ovary and mammary glands (more than 90% cells), and its loss has been correlated with high tumour grade (Woelfle, Sauter et al. 2004; Kurman and Shih Ie 2010). A similar observation is seen with cisplatin treated PA1 cells whereby the cancer stem cell related markers NESTIN, SOX2, NANOG, OCT4, CD44, CD133 CD49f and SSEA4 were down-regulated (47.4%, 58.3%, 59.3%, 81.6%, 48%, 84.7%, 41.2% and 66.7%), whilst differentiation markers CK18, CD24 and β-tubulin were significantly up-regulated (90%, 180% and 192% respectively) (Suppl. Table 3). Expression of SMA, a basal/myoepithelial differentiation marker was interestingly up-regulated (12%) (Suppl. Table 3) with cisplatin which may be a novel finding which shows cisplatin induces multi-lineage differentiation whereby a switch from one phenotype (luminal epithelial) to another phenotype (basal) is made possible. A similar observation was shown by (Prabhakaran, Hassiotou et al. 2013) where cisplatin induced a basal to epithelial phenotypic switch in MDA-MB-231 cells, an aggressive TNBC. The only difference was in the amount of up and/or down regulation of these proteins showing different target preference.

The more interesting and also novel finding in this study was the synergistic effect of SOX2 iPep and cisplatin in PA1 cells. SOX2 (99.2%), SSEA4 (97.5%) CD133 (95.8%) ki67 (95.4%) and OCT4 (93%) expression was dramatically down-regulated whilst CD24 (240%), β-tubulin (243.7%) and CK18 (149.1%) expression was dramatically increased (Suppl. Table 3). These findings clearly show that SOX2 inhibition/knockdown in combination of cisplatin treatment enhanced the anti-cancer effect of cisplatin and influenced gene expression by inhibiting the oncogenic function in the PA1 cancer cells and shifting them towards a more differentiated phenotype that has lost its proliferative capacity. A similar finding was shown by Du and co-workers whereby knockdown of NANOG using a siRNA in combination with cisplatin improved the chemosensitivity of esophageal cancer to chemotherapy (Du, Shi et al. 2012). The existing anti-cancer drug therapies are experiencing a huge shift from cytotoxic-based to differentiation-inducing therapies, as many types of tumours undergo further acquired resistance, recur and/or metastasise after treatment due to survival of the resistant CSCs
The main interest of tumour cell differentiation therapy is to induce maturation of the less differentiated CSCs into specific lineages that are differentiated, which in turn reduces proliferation capacity and tumorigeneicity (Hadnagy, Beaulieu et al. 2008). Although the mechanism of action of both SOX2 iPep and cisplatin is focused on the DNA, each drug possesses different specificity in targeting CSCs. This is observed through the cytotoxicity of the iPep in SOX2 expressing tumour cells, which suggest differentiation induction through the specific down-regulation/inhibition of SOX2. It is postulated that tumours with abundant SOX2 mRNA levels are more sensitive to the iPep compared to low or non-expressing tumours. Cisplatin on the other hand showed cytotoxic effects regardless of SOX2 expression and yet induced differentiation, for example in the poorly differentiated MDA-MB-231 TNBC cells (Prabhakaran, Hassiotou et al. 2013). Another difference between the drugs is that the cisplatin binds and form adducts in the major groove of DNA, while the SOX2 iPep is believed to bind in the minor groove of the DNA where the HMG proteins reside (Ohndorf, Rould et al. 1999; Hurley 2002; D'Angelo, Borbone et al. 2013). The different binding sites of cisplatin and SOX2 iPep on the DNA may result in regulation of different target proteins. Apart from that, it is very interesting how these two drugs could work in synergy. The binding and adduct formation of cisplatin on the DNA double helix (Zeidan, Jenkins et al. 2008; Basu and Krishnamurthy 2010; Florea and Busselberg 2011; Wang, Milum et al. 2011) as well as nuclear localization of the iPep and nuclear SOX2 TF function is predicted to work in unison in an unknown mechanism aiming towards differentiation of tumour cells.

I propose that the interaction and formation of SOX2 iPep-cisplatin complex in the nucleus post internalization, binds DNA and causes conformational change, which in turn opens up the DNA double helix. This can be explained by the cisplatin crosslinking at d(GpG) on the DNA in the major groove which causes it to bend and unwind the duplex and the altered structure attracts HMG proteins such as SOX2. This unwinding of the double helix render improved accessibility of the iPep-cisplatin complex to the SOX2 promoter region/target specific site in the minor groove of DNA (Ohndorf, Rould et al. 1999; Suntharalingam, Mendoza et al. 2013) whereby SOX2 function is eventually inhibited as the interaction of SOX2 with the SOX2-iPep-cisplatin complex prevents interactions of other TFs such as OCT4 and PAX6.
CHAPTER 5: SOX2 TARGETED TREATMENT IN CANCER

Another possible way these drugs could work in synergy to enhance their anti-cancer effects is by completely blocking the DNA damage repair network, for example the nuclear excision repair mechanism (NER) which is a major drawback of cisplatin treatment. CSCs and hESCs share similar characteristics (Hassiotou and Geddes 2012; Hassiotou, Hepworth et al. 2013; Rocha, Lerner et al. 2013) which also includes the DNA repair mechanism (Hyka-Nouspikel, Desmarais et al. 2012; Rocha, Lerner et al. 2013). The main role of the DNA repair mechanism is to overcome DNA lesions and preserve genomic integrity, which is essential for pluripotent cells due to their self-renewal and cell maintenance characteristics (Rocha, Lerner et al. 2013). The iPep-SOX2 protein interaction and binding to cisplatin molecule could lead to bulky and strong adduct formation on the DNA where the major and minor grooves meet (Lange, Takata et al. 2011). This distorting bulky adducts are able to conceal the DNA lesion formed by cisplatin and are protected from the DNA damage repair machinery (Hurley 2002; Lange, Takata et al. 2011). The unrepaired cisplatin-DNA lesions induced cancer cell death or differentiation as response to DNA damage (Hyka-Nouspikel, Desmarais et al. 2012; Rocha, Lerner et al. 2013). The synergistic effect of cisplatin and SOX2 iPep is predicted to sensitize chemotherapy through a combined transcriptional control of tumour cells. Fig. 7 illustrates a proposed model based on our findings, which depicts the role of SOX2 iPep in the differentiation of SOX2-enriched cancer cell lines.

Future studies should focus on confirming the specific targeting and inhibition of SOX2 via the iPep. This can be done by conducting a SOX2 rescue experiment through the insertion of SOX2 cDNA, similar to the work done relating to the specific targeting of EN1 (Beltran, Graves et al. 2013). Insertion of the cDNA should reverse the inhibitory effect and revive cell survival of the cancer cells, while shRNA knockdown against SOX2 would to confirming that the SOX2 iPep effect is on-target. Subsequently, it will be important to determine the binding partners of SOX2 TF. Next, regulation of similar markers examined at the protein level should be further investigated at the gene level via (chIP) sequencing. It would also be of great interest to conduct RNA sequencing to check for global transcriptomal changes caused by the SOX2 inhibition. Lastly this work should be extended to additional ovarian cancer and TNBC lines with elevated SOX2 expression.
Figure 7. A proposed model of SOX2 iPep mechanism of action in SOX2 overexpressing cancer cells and how it influences the cellular hierarchy. The diagram illustrates a possible mechanism of SOX2 iPep effects in SOX2 overexpressing cancer cells. Cancer stem cells can differentiate, potentially by the knockdown of SOX2 TF, into a more mature epithelial phenotype, luminal or myoepithelial, which has lost or possesses limited proliferative potential. As the more differentiated cells are more susceptible to chemotherapy, this push of differentiation may assist in the management and/or treatment of cancer.

5.6 Conclusions
SOX2 is an important TF and key regulator of pluripotency and self-renewal in embryonic stem cells. Overexpression of SOX2 in normal cells leads to tumorigenesis through the generation of CSCs and has become the basis of several cancers such as TNBCs, (SOC and brain cancer. As high SOX2 gene and protein expression is often linked to aggressive tumours, poor prognosis and lowest survival rates, it is crucial to develop new anti-cancer therapies that specifically target the CSC population within the tumour by the tackling of transcription machinery. The unique and novel property of the SOX2 iPep to block protein-protein interactions between SOX2 and its important binding partners reduced cell viability significantly in SOX2 expressing tumour cells while no harm was seen in normal epithelial and very low/no-SOX2 expressing tumour cells, indicating high specificity of the iPep. Moreover, the iPep also reduced cell proliferation, down-regulated SOX2 and NANOG, its binding partners as well as other stem cell markers in PA1 SOC cells and induced differentiation. In addition to that, the iPep also interestingly synergized with cisplatin and further enhanced the anti-cancer effect in PA1 and T11 (TNBC) cells by reducing the drug dose, which is a major advantage of a combinatorial chemotherapy treatment.
CHAPTER 6

GENERAL DISCUSSION
CHAPTER 6: GENERAL DISCUSSION

Breast cancer affects more than 1 million women worldwide. About 400,000 patients die due to this disease annually (Kutomi, Ohmura et al. 2012; Sun, Zhang et al. 2012). The TNBC subtype which is distinguished by the lack of ER, (PR and HER2 receptors represents about 16% of all breast cancers (Shah, Roth et al. 2012) The basal-like and claudin-low subgroups make up the TNBC subtype, which is known for its poor prognosis and worst survival rate due to high relapse frequency among patients apart from being highly proliferative and metastatic (Carey, Dees et al. 2007; Prat, Parker et al. 2010). The basal-like and claudin-low tumours are enriched with CSCs. In addition to that, the claudin-low possess epithelial-to-mesenchymal transition (EMT) features and immune system responses (Prat, Parker et al. 2010; Prat and Perou 2011) making them the most aggressive breast tumours. Apart from that, the claudin-low tumours have an intermediate chemotherapy response rate between that of basal-like and luminal tumours (Prat, Parker et al. 2010; Prat and Perou 2011). The only available and effective chemotherapy for the TNBCs is the platinum-based drugs, especially cisplatin. Although the basal-like and claudin-low are more sensitive to chemotherapy compared to luminal breast tumours, these tumours are prone to acquired resistance, while the luminal tumours are often effectively treated with hormonal-based targeted therapies. It is indeed a big challenge to treat patients with TNBCs as there are no specific targeted therapies for this highly aggressive disease. Based on these challenges, the work done in this thesis revolves on the characteristics of promising treatments of TNBCs. Most of the breast cancer cell lines used in this project represent the claudin-low TNBCs such as BT-549, MDA-MB-231, MDA-MB-435 and SUM-149 that are human lines, while T11 is a mouse line (Prat and Perou 2011). Basal-like MDA-MB-468 and luminal MCF-7 cell lines (Prat and Perou 2011) were also used.

In all the chapters, the drug incubation period is between 8 to 24 hours although most studies show between 24 to 72 hours in cancer treatment. This time frame was chosen mainly because it would have been difficult to see or evaluate differentiation induced by cisplatin if treated for a longer period of time as the cells would be mostly dead or in contrast regained its self-renewal property post 24 hours while at a shorter period of treatment, cisplatin will not function sufficiently. In chapter 4 and 5, since the iPeps used have short half lives, the incubation time was limited as the iPeps would be no longer in existence to show its effect. Most importantly, prominent cellular as well as molecular and in addition morphological changes were observed within these drug incubation period.
In chapter 3, the main focus was on the anti-cancer effect of cisplatin as a single agent in a panel of breast cancer cell lines, including TNBC cells. As it is already known that TNBCs are sensitive to cisplatin treatment, it was interesting to investigate the factor(s) associated with this sensitivity. The most commonly known fact on cisplatin is that it forms cytotoxic adducts with the DNA causing tumour cell death. However, not much is known about the specific cancer cell toxicity mechanisms of cisplatin action, which can be tumour specific. Based on the fact that TNBCs are enriched with CSCs and very proliferative, I hypothesised that the CSC subpopulation could be an important target of cisplatin although there are no previous works showing its ability to specifically target CSCs. A cellular hierarchy consisting of cells with stem cell properties to more differentiated cells were observed in MCF-7, MDA-MB-468, MDA-MB-231 and BT-549 cells in different proportions. MCF-7 was most differentiated, followed by a decreasing differentiation state of MDA-MB-468, BT-549 and MDA-MB-231, which was the least differentiated. In contrast, CD49f and SSEA4 stem cell markers were highly expressed in BT-549 and MDA-MB-231 cells. Treating these cells with clinically used cisplatin doses interestingly induced cell differentiation evident by the down-regulation of stem cell markers and up-regulation of differentiation markers. This finding proposes a novel and possible mechanism of action of cisplatin in aiming CSCs-mediated metastases and tumorigenesis. This push of differentiation may assist in the future treatment of breast cancer as differentiated tumour cells are more susceptible to chemotherapy. Tumour cells enriched with CSCs can be first treated with cisplatin followed by another drug/agent that targets differentiated cells. In spite of this promising benefit, cisplatin as a single agent needs a sufficient amount of dose to be administered and be effective in patients, however 20 µM of cisplatin and above is often correlated with severe side effects in patients. Furthermore, the risk of acquired resistance still remains a challenge as cisplatin commonly is known to increases the proportion of CSCs within the tumour with treatment in time (Al-Ejah, Smart et al. 2011; Kallifatidis, Labsch et al. 2011; Abada and Howell 2014). Cisplatin sensitivity in CSC-enriched cancers may be short termed which may explain the initial positive outcome in patients but become resistant after one or two treatments. Regeneration of the persisting CSC residue from the cisplatin treatment is believed to result in increased mass of tumour. In order to overcome the resistance to cisplatin, a slightly shorter treatment period could be used but in conjunction with a second or third therapeutic agent which could preserve the potent anti-cancer properties of cisplatin subsequently
prevent possible de-differentiation of cancer cells into CSCs and metastases (Kallifatidis, Labsch et al. 2011; Baribeau, Chaudhry et al. 2014; Vassilopoulos, Xiao et al. 2014). Besides that, minimizing cisplatin toxic side effects is also an important factor, and the only possible option is by using lower cisplatin dose in combination with other possible therapeutic agents which will still maintain and/or enhance the anti-tumour effect of cisplatin without harming normal cells.

Chapter 4 therefore aimed to examine the effect of specific transcription factor (TF) targeted inhibition via an iPep in SUM-149 and T11 cells along with cisplatin. This project was established based on the recent findings by our laboratory demonstrating EN1 oncogene overexpression in SUM-149 and T11 cells and that specific targeted inhibition of the EN1 TF using a novel EN1 iPep (Beltran, Graves et al. 2013) induced apoptosis in tumour cells. The EN1 is a key regulator of the early embryonic developmental stage in the central nervous system. Initially a combinatorial treatment with the EN1 iPep and cisplatin in SUM-149 cells was conducted to investigate the possibility of a synergistic or additive effect of both the agents. The inhibition of EN1 via iPep did not sensitize the TNBC cells to cisplatin, although the EN1 iPep and cisplatin as single agents showed potent anti-cancer effects. It may be possible that cisplatin is not involved in inflammatory pathways while EN1 iPep does, suggesting that the targeted EN1 inhibition via iPep could be a potent anti-cancer therapy in treating TN-IBCs. This led to further investigation of the possible pathway involved in the anti-cancer properties induced by EN1 specific targeted inhibition in SUM-149 cells. The findings in this chapter are novel, significant and give insight on the relationship between the EN1 iPep, EPRS, inflammation and tumorigenesis in TNBC cells. A co-localization of EPRS and the EN1 iPep substantiated an interaction between them, which is assumed to control the level of inflammatory substances in the tumour cells. Chronic inflammation in cancers has often been correlated to poor prognosis and worst survival rate. Hence identification of therapeutic agents that could reduce this inflammation in cancer is crucial. The findings in this chapter reveal the potential of EN1 iPep as a potent anti-inflammatory therapeutic agent in breast cancer and possibly in other immune-compromised diseases. Proline-enriched and an EPRS target COLLAGEN and CD69 pro-inflammatory genes were down-regulated while pro-inflammatory IL11 and tumour suppressor FOXA2 genes were up-regulated. Furthermore, a down-regulation in proline enriched COLLAGEN suggested the iPep may compete with proline by binding at the active site on EPRS to induce anti-
inflammatory properties. This led to the radioactive proline incorporation assay to examine the specificity of the iPep in competing with proline binding site. Methionine as a control was also examined. Interestingly, a reduction in proline incorporation in EN1 iPep treated SUM-149 cells compared to the mock cells was observed, while methionine incorporation was not affected upon treatment. To further confirm this finding, proline and methionine were respectively added in excess to the iPep treated cells to see if this addition rescued to SUM-149-iPep treated cell. As expected, proline at 50 µM increased the cell viability, while methionine up to 50 µM had no effect on cell proliferation. Hence, it was revealed that EN1 targeted inhibition via a specific EN1 iPep controls inflammation in TN-IBC through the EPRS pathway by competing with the proline active site.

The promising anti-cancer effect of the EN1 iPep in treating inflammatory based TNBC cells by targeting the overexpressed EN1 TF formed the basis for Chapter 5 of this thesis. A second iPep which specifically targeted SOX2 was designed and constructed similar to EN1. SOX2 is an important key regulator of cell self-renewal and pluripotency, and is overexpressed in TNBCs and serous ovarian carcinoma (SOC) cells. Both TNBCs and SOCs share various characteristics in addition to SOX2 overexpression such as BRCA1 mutation, CSC enrichment are highly proliferative and metastatic, have poor prognosis and the worst survival rates among all breast and ovarian cancers respectively. Most importantly, they are both sensitive to cisplatin treatment. As Chapter 3 revealed a new mechanism of cisplatin action in inducing differentiation of TNBCs, the main aim of Chapter 5 was to investigate if the specific SOX2 targeted inhibition sensitised the cancer cells to cisplatin. SOX2 targeted inhibition was chosen because it is known that SOX2 expression is lost upon cell differentiation (Lopez, Valdez-Morales et al. 2013). Since both cisplatin and SOX2 inhibition via iPep are involved in differentiation, it is hypothetical that these agents may work together. T11 mouse mammary carcinoma cells representing the claudin-low subtype and PA1 SOC cells were mainly studied because these cells demonstrate the highest SOX2 mRNA levels. The main finding in this chapter was that the inhibition of SOX2 via SOX2 iPep synergised with cisplatin and sensitised both T11 (TNBC) and PA1 (SOC) cells to cisplatin treatment, reducing the IC_{50} values from 16.01 µM to 3.51 µM in T11 cells, and 16.25 µM to 4.39 µM in PA1 cells. In addition to that, using very small dose of cisplatin and also the iPep could reduce the risk of adverse side effects caused by cisplatin, which subsequently could improve the quality of life in these
patients. This promising finding led us to investigate whether these agents in single form and in combination induce differentiation in PA1 cells. This cell line was chosen as it expresses the highest SOX2 levels among the panel of cell lines tested. Similar to cisplatin induced differentiation in TNBCs (Prabhakaran, Hassiotou et al. 2013) (Chapter 3), PA1 ovarian cancer cells also differentiated upon cisplatin by significant down-regulation of SOX2 and various other stem cell proteins including ki67, a proliferation marker, and by up-regulation of differentiation markers, especially the epithelial cell marker CK18. The cisplatin-SOX2 iPep combinatorial treatment almost completely inhibited cell viability, SOX2 and ki67 expression. These findings are vitally important and now form the basis for the new promising combinatorial treatments for TNBC and SOC patients.

In conclusion, the studies done in this thesis cover a small but specific field of cancer biology in relation to treatments in TNBCs and in addition, SOCs. The findings in this thesis provide novel grounds for new effective treatment options for the most aggressive cancers affecting women worldwide. Apart from revealing a new cisplatin mechanism of action, EN1 and SOX2 are also highlighted as important genes that can be easily and specifically targeted via interference peptide technology. The data of this thesis provide strong evidence for the efficacy of this technology in specific targeted cancer therapies that can act synergistically with current potent anti-cancer drugs and can aid the management of drug targeting and delivery in treating cancers and potentially other diseases.


Mayor, R., L. Casadome, et al. (2009). "Long-range epigenetic silencing at 2q14.2 affects most human colorectal cancers and may have application as a non-invasive biomarker of disease." Br J Cancer 100(10): 1534-1539.


**TABLE S1. Antibodies used.**

<table>
<thead>
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<th>Antibody</th>
<th>Clone</th>
<th>Cat. Number</th>
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<th>Application</th>
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<td>3k1</td>
<td>09-0045</td>
<td>STEMGENT</td>
<td>FC: 1:50</td>
</tr>
<tr>
<td>CK18</td>
<td>CY90</td>
<td>MCA1864H</td>
<td>AbA Serotech.</td>
<td>FC: 100 µL, IF: 100 µL</td>
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<tr>
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<td>Santa Cruz Biot.</td>
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<td>-</td>
<td>Hybridoma</td>
<td>-</td>
<td>FC: 100 µL, IF: 100 µL</td>
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<tr>
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<td>09-0006</td>
<td>STEMGENT</td>
<td>FC: 1:50</td>
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<td>Integrin-α6 (CD49f)</td>
<td>GoH3</td>
<td>555735</td>
<td>BD Pharmingen™</td>
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<tr>
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<td>-</td>
<td>Ab833</td>
<td>Abcam,</td>
<td>FC: 1:100 IF: 1:800</td>
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<td>Cell type</td>
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TABLE S3. PA1 ovarian carcinoma cell characterisation and effects of SOX2 iPep, cisplatin and SOX2 iPep+cisplatin combination on protein expression.

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<th>Treatment condition</th>
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<th>Markers</th>
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<th>After treatment (MFI)</th>
<th>Up/down regulation (%)</th>
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<td>4.2</td>
<td>↓ (58.6)</td>
</tr>
<tr>
<td></td>
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<td>5.1</td>
<td>4.3</td>
<td>↓ (15.7)</td>
</tr>
<tr>
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<td>CD24</td>
<td>3.2</td>
<td>10.4</td>
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</tr>
<tr>
<td></td>
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<td>3.5</td>
<td>↓ (64.3)</td>
</tr>
<tr>
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<td>38.1</td>
<td>4.6</td>
<td>↓ (88)</td>
</tr>
<tr>
<td></td>
<td></td>
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<td>30.7</td>
<td>4.7</td>
<td>↓ (84.7)</td>
</tr>
<tr>
<td></td>
<td></td>
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<td>↓ (18.4)</td>
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<td>35.1</td>
<td>9.9</td>
<td>↓ (71.8)</td>
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<tr>
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<td>5.6</td>
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</tr>
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<td>Stem cells</td>
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<td>------------------</td>
<td>----------------------</td>
<td>-----------------------------------</td>
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<td>243.4</td>
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VIDEO: SOX2 iPep internalization in T11 cells