School of Biomedical Sciences

The Role of Secreted Frizzled-related Protein-4 (sFRP4) in the Epigenetics, Metabolism and Chemo-sensitisation of Cancer Stem Cells

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Doctor of Philosophy
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Declaration

To the best of my knowledge and belief this thesis contains no material previously published by any other person except where due acknowledgment has been made.

This thesis contains no material which has been accepted for the award of any other degree or diploma in any university.

Signature: 

Date: 06th November 2017

Dedication

I dedicate this work to my parents, P.S and Alka, my two sisters, Pooja and Sweta, and all my friends who offered me unconditional love and support and have always been there for me. Thank You so much.

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List of Publications

This thesis is assembled by publications (either published or submitted) which form the individual chapters of the thesis.
The publications are listed as follows:

Chapter 1

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Chapter 2

Deshmukh, A., Kumar, S., Arfuso, F., Newsholme, P., & Dharmarajan, A. (2017). Secreted Frizzled-related protein 4 (sFRP4) chemo-sensitizes cancer stem cells derived from human breast, prostate, and ovary tumor cell lines. Sci Rep 2017 May23; 7(1): 2256.
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Chapter 3

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Chapter 4

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Chapter 5

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Statement of Contribution by Others

I hereby declare that the work presented in this thesis was primarily designed, experimentally executed, interpreted, and written by the first author of the individual manuscripts (Abhijeet P. Deshmukh). Contributions by colleagues are described in the following. The signed statement by co-authors is in Appendix 2.

Chapter 1

1. Abhijeet Deshmukh drafted the outline and generated the figure. Abhijeet Deshmukh wrote the manuscript. Mudra Binju contributed to the manuscript. Abhijeet Deshmukh conceived of the study and Arun Dharmarajan, Frank Arfuso, and Philip Newsholme critically reviewed, revised, and approved the final manuscript.

2. Abhijeet Deshmukh drafted the outline and generated the figure. Abhijeet Deshmukh wrote the manuscript. Kedar Deshpande contributed to the manuscript. Abhijeet Deshmukh conceived of the study and Arun Dharmarajan, Frank Arfuso, and Philip Newsholme critically reviewed, revised, and approved the final manuscript.

Chapter 2

Abhijeet Deshmukh drafted the outline and generated the figure. Abhijeet Deshmukh wrote the manuscript. Senthil Kumar contributed the viability data. Abhijeet Deshmukh conceived of the study and Arun Dharmarajan, Frank Arfuso, and Philip Newsholme critically reviewed, revised, and approved the final manuscript.

Chapter 3

Abhijeet Deshmukh drafted the outline and generated the figure. Abhijeet Deshmukh wrote the manuscript. Senthil Kumar contributed the viability data. Abhijeet Deshmukh conceived of the study and Arun Dharmarajan, Frank Arfuso, and Philip Newsholme critically reviewed, revised, and approved the final manuscript.

Chapter 4

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Abstract

Cancer stem cells (CSCs) are known to be highly chemo-resistant and, in recent years, have gained intense interest as key tumour-initiating cells that play an integral role in cancer recurrence following chemotherapy. Wnt/β-catenin signalling is important for proliferation and self-renewal of CSCs in association with human tumorigenesis. This study investigated molecular signals essential to sustain CSCs and how to target their activity using secreted frizzled-related protein 4 (sFRP4) alone or in combination with chemotherapeutic drugs. We also investigated the possible involvement of methylation-mediated silencing of the sFRP gene family in CSCs derived from breast, prostate, and ovary tumour cells lines. Expression of sFRPs has been found to be downregulated in various cancers, and is associated with disease progression and poor prognosis. During tumour development, sFRP genes are hypermethylated, causing transcriptional silencing. SFRPs have an ability to sensitize tumour cells to chemotherapeutic drugs, thereby enhancing cell death. Since Wnt signalling promotes glycolysis and tumour growth we investigated the effect of the Wnt antagonist sFRP4 on CSC metabolism. We also demonstrated that sFRP4 has a prominent role in basal glucose uptake in CSCs derived from breast and prostate tumour cells. We showed that sFRP4 treatment on CSCs isolated with variable glucose content induced metabolic reprogramming by relocating metabolic flux to glycolysis or oxidative phosphorylation. Altogether, sFRP4 treatment compromises the tumour cell proliferation, and critically affects the cell survival mechanisms as associated with viability, glucose transporters, pyruvate conversion, mammalian target of rapamycin (mTOR), while promoting CSC apoptosis under conditions of variable glucose content. Furthermore, we demonstrated the association between aberrant Wnt signalling and CSCs. Hence, treatment of CSCs with sFRP4 would enable their chemo-sensitisation and improve chemotherapeutic efficacy in the clinical setting and will also provide the feasibility of using sFRP4 to inhibit CSC survival in order to induce metabolic reprogramming *in vivo.*

Table of Content

Chapter 1

Deshmukh, A., Binju, M., Arfuso, F., Newsholme, P., & Dharmarajan, A. (2017). Role of epigenetic modulation in cancer stem cell fate. *The International Journal of Biochemistry & Cell Biology*, *90*, 9-16.

See appendix 1 for the published paper

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See appendix 3 for the published paper

1 Literature Review

1.1 The Role of epigenetic modulations in cancer stem cell fate

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Keywords: cancer stem cells, epigenetics, Wnt signalling pathways, DNA methylation, Histone modification, chromatin remodeling, Polycomb gene, chromatin immunoprecipitation

Abbreviations

CSCs: Cancer stem cells; PcG: Polycomb gene; PRC: Polycomb repressive complex; CpG: *5′-C-phosphate-G-3′*; DNMT: DNA methyltransferase; EZH2: Enhancer zeste homologue 2; Hh: Hedgehog; MSP: Methylation specific PCR; HAT: Histone acetyltransferase; HDAC: Histone deacetylase; HMT: Histone methyltransferases; HDM: Histone demethylases; ChIP: Chromatin immunoprecipitation.

Abstract

A sub-population of the tumor micro-environment consists of cancer stem cells (CSCs), which are responsible for the initiation and recurrence of cancer. Recently, epigenetic processes such as DNA methylation, histone modification, and chromatin remodelling have been found to be involved in inducing epigenetic factors in CSCs. Most of these processes, such as DNA methylation, generally occur in the genome that is rich in Cytosine-Guanine repeat sequences, also known as CpG islands, which are distributed throughout the human genome. The Polycomb gene (PcG) complex is a chromatin modifier facilitating the maintenance of embryonic and adult stem cells. Recent evidence suggests that the PcG is also involved in maintaining CSC stemness. We have presented various aspects and examples of how epigenetic modulations may drive or promote tumorigenesis and metastasis by alteration of key transcriptomic programs and signalling pathways in CSCs.

1.1.1 Introduction

The cancer microenvironment consists of some highly active cells that possess stem cell-like properties including a high proliferation rate and differentiation potency; hence they have been referred to as cancer stem cells (CSCs) (1). Some of the unique properties of CSCs include self-renewal and altered response to stress conditions (2, 3). CSCs are a subset of the tumor cell population that possess high regenerative characteristics, metastatic capacity, sphere forming ability, and high ABC drug efflux transport systems (4, 5). CSCs help maintain the cancer environment by continuously differentiating into cancer cells; however, their origin is still a subject of controversy. Just like other adult stem cells in mammals, CSCs are also found to be arrested in a quiescent state, i.e. G0 phase (6, 7). This state is however, reversible, by differentiation of CSCs into non-stem cancer cells that can further divide (8). Therefore, recent studies have focussed on targeting CSCs to reduce the tumor bulk in order to eradicate cancer (9).

CSCs are enriched with various growth factors including epidermal growth factor (10) and fibroblast growth factor (FGF) that are involved in maintaining their stem-cell properties (11, 12). CSCs possess 4 transcription factors that help in maintaining the stem cell-like properties, which are Octomer-binding transcription factor 4 (Oct4)(13), Nanog homebox (NANOG)(14), Krupple-like factor 4 (Klf4)(15), and Sex determining region Y box-2 (Sox2)(16). CSCs also express specific surface markers, for example - CD44 is the most common CSC surface marker for colon, gastric, breast, pancreatic, ovarian, and head/neck cancers; thus it is being targeted for cancer treatment therapies. Various examples of CSC surface markers are listed in Table 1.

Table 1: Tumor specific CSC Markers

Type Of Cancer	CSC markers	Reference
Pancreatic cancer	CD133+/CXCR4+, CD24+/CD44+, c-Met+/CD44+, CD24+/CD44+/ESA+, and ALDH1+/CD133+	(12)
Leukemia	CD34+/CD38-	(17)
Liver cancer	CD133/CD45/CD90	(18, 19)
Malignant Melanoma	CD20/CD166/Nestin	(20)
Glioblastoma	CD133+/ABCG2+	(21)
Lung cancer	CD117/CD90/EpCAM	(22)
Breast cancer	CD24-/low/CD44+, ALDH1	(11, 23)

1.1.2 Epigenetics in CSCs

CSCs undergo similar genetic processes to normal stem cells, such as DNA methylation and chromatin remodeling. To understand the processes of self-renewal, differentiation, and proliferation in CSCs, the molecular pathways associated with epigenetics and gene regulation have been extensively examined (24, 25). DNA methyltransferase 1 (DNMT 1) regulates DNA methylation in human genes and is further maintained by DNMT3A and DNMT3B (26). In a study involving a leukemic murine model, wild-type mice with DNMT1 overexpression developed leukaemia while DNMT1 knock-out mice ($DNMT1^{-/-}$) were free from the disease (27, 28). Loss of DNMT1 expression resulted in subsequent loss of genomic stability, cellular functions just as differentiation and viability of cellular function, are characteristics noted in most of malignant process (29, 30). The possible explanation proposed for this finding is that there is hypo-methylation of tumor suppressor genes. Further, a chromatin immune-precipitation (CHIP) assay was performed with Histone-3 lysine 27 (H3K27me23) or H3 antibodies (27, 28). The result showed suppression of Enhancer Zeste Homologue 2 (EZH2)-regulated target genes in DNMT1, indicating that the Polycomb gene (PcG) complex contributes to DNA methylation by regulating leukemia stem cells (27, 28, 31). PcG is an epigenetic modifier that is involved in the encoding of epigenetic silencers (32).

1.1.3 Wnt signalling pathway and CSC epigenetics

The Wnt signalling pathway is a crucial regulator of various cell activities including differentiation, proliferation, self-renewal, and cell movement (33). Research shows that any aberration in this pathway can lead to tumor progression (34, 35). Wnts consist of 19 glycoproteins that can bind to cell surface receptors with different combinations (36, 37). In canonical pathways, the Frizzled transmembrane receptor and low-density lipoprotein receptor-related protein (LRP5/6) transduce Wnt signalling by stimulating β-catenin, a critical protein that regulates growth-promoting genes (33, 36). The pathway is co-regulated by various positive and negative regulators; loss of negative regulators thereby leads to upregulation of gene transcription and results in tumor development (38-41).

Wnt binding to Frizzled causes aggregation of Dishevelled (Dvl) to form a scaffold-like structure at the plasma membrane (42). This structure stimulates clusters of LRP5/6 that are phosphorylated by casein kinase I, followed by association of Axin, glycogen synthase kinase

3-β (GSK-3-β), and adenomatous polyposis coli (APC) to LRP singlosomes (42). This inhibits the activity of GSK-3-β and accumulation of β-catenin in the cytoplasm (43).

Recently, methylation of the promoters of genes involved in the Wnt signalling pathway has been identified in the case of breast cancer (38, 44-46). Wnt inhibitory factor-1 (WIF1) is a Wnt-binding protein that acts as a tumor suppressor and is also found to be downregulated in many tumors such as prostate, lung, and skin cancers (46). Methylation-specific PCR revealed hypermethylation of WIF1 in 16 of 24 tumor tissue samples (44). Furthermore, DNMT1 and DNMT3-β work in a synchronised fashion to cause methylation of WIF1 (44).

The secreted frizzled-related protein (sFRP 1 to 5) family acts as a Wnt antagonist. sFRP1 is mostly silent in many tumor types (47). Real-time PCR analysis shows that sFRP1, 2, and 5 are methylated and downregulated in breast cancer cell lines (45).

Various agents are being tested as Wnt pathway inhibitors, such as nonsteroidal anti-inflammatory drugs and thiazolidinedione (an antidiabetic agent), which have already been approved for therapeutic use and are under clinical trials (48-50). Two monoclonal antibodies (Wnt ligand neutralizers and Wnt receptor Fz and LRP inhibitors) that target Wnt signalling are also in clinical trials (51).

1.1.3.1 Epigenetics in relation to sFRP4

It has been observed that WNT/β-catenin/GSK-3 signalling is important for proliferation and self-renewal of normal and CSCs due to its multiple genetic alterations in association with human tumorigenesis, which has been shown in medulloblastoma, hepatocellular cancer, and leukemia (52).

In previous studies, the sFRP family has shown the ability to chemo-sensitize various tumor cells to treatment (53), resulting in a decreased proliferation rate and increased induction of apoptosis, particularly during tumor development when the cells are hypermethylated and inducing transcriptional silencing (54, 55). In cancers such as glioblastoma multiforme, the down-regulation and gene-silencing of the sFRP family through epigenetic modification has been demonstrated (56).

Secreted frizzled-related protein 4 (sFRP4) is a Wnt antagonist (57). In a recent study, sFRP4 was used as a biomarker for colorectal cancer risk (58). It has also been reported that

epigenetic events cause methylation of CpG islands, resulting in the down-regulation of sFRP4 (56). Thus, the Wnt pathway is not obstructed, resulting in tumorigenesis.

Many studies suggest that various signalling pathways are also responsible for the maintenance and survival of CSCs (59). Examples of such signalling pathways include – Wnt/β-catenin, Hedgehog, and Notch (59). All of these pathways are involved in the development and growth of cells in the body under normal circumstances; however, one or more aberrations can give rise to cancer (59).

1.1.4 Notch signalling pathways and CSC epigenetics

Like Wnt, the Notch signalling pathway is conserved during eukaryotic phylogeny and plays a role in regulating embryonic and adult stem cell fate in mammals (60, 61). The Notch pathway's role in CSC survival could be related to the activation of tyrosine kinase PKB (62), a well-known mechanism to escape cell death and initiate chemo-resistance. Furthermore, the Notch target gene *IL6* mediates drug resistance in prostate cancer cells (63). The epigenetic regulation of genes could be involved in various CSC pathways, such as p300 mediating the activation of NF-$_{\kappa}$B by signal transducer and activation of transcription 3 (STAT3) (64). Activation of NF-$_{\kappa}$B leads to stimulation of IL6 production, which in turn activates STAT3. IL6 plays an important role in CSC chemo-resistance and self-renewal capacity (65). Also, inhibition of the Notch signalling pathway depletes pancreatic progenitor cells, which exhibit similar to phenotypic characteristics to CSCs (66, 67). However, induced Notch activation prevented epithelial differentiation, resulting in increased maintenance of undifferentiated pancreatic progenitor cells (68). The Notch pathway also helps in maintaining the population of CSCs in the tumor microenvironment. Thus, this pathway can be a therapeutic target for cancer treatment (69).

Deregulation of Notch signalling has been found to be common to many tumors (61). It involves communication between contiguous cells via transmembrane ligands and receptors (70, 71). This inter-cellular communication triggers proteolytic cleavage of the receptor, which in turn releases an intracellular fragment that regulates target gene expression (72). The pathway involves five types of ligands – Delta-like ligand (DDL) 1, 3, and 4, and Jagged1 and Jagged2, as well as four receptors (Notch 1 to 4) (72). A recent study suggests that DLL1 is regulated by epigenetic processes in gastric cancer (61). In this study, the promoter of

DLL1 was found to be unmethylated in a mouse model of gastrointestinal cancer (61). It has been reported that hypermethylation of the DLL1 promoter leads to its silencing and repression of Notch signalling in gastric cancer (61). The data linking the Notch signalling pathway and epigenetic deregulation in CSCs are not well established, though we could elucidate the role of Hes proteins, which could activate or inhibit sets of genes by recruiting histone acetylates and deacetylase (73). A deeper dissection of the Notch-Hes pathway would enable the further role of Hes targets, such as p53 inhibitor mdm2 and Gata transcription factors, to be unveiled.

1.1.5 Hedgehog signalling pathway and CSC epigenetics

The Hedgehog (Hh) signalling pathway is mainly involved in tissue repair, embryonic development, and epithelial-to-mesenchymal transition of cells (74). Hh ligands involved in signalling - Sonic Hedgehog (Shh), Indian Hedgehog (Ihh), and Desert Hedgehog (Dhh), undergo cleavage and produce a signalling protein with dual lipid modifications (75, 76). In mammals, the three mechanisms that can cause mutations in the Hedgehog pathway include ligand-independent and ligand-dependent, excessive expression of Hh ligands, and the generation of a CSC phenotype (77). Hedgehog molecules are paracrine molecules that bind to patched (PTCH) receptors, which in turn activate smoothened (SMOH) and translocate glioma associated homolog (GLI)-1, 2, and 3 to the nucleus to modulate gene expression. This signal transduction pathway is required for self-renewal and proliferation of cerebellar, retinal, and pancreatic CSCs (78). Furthermore, GLI transcription activates *BMI1*. The hedgehog-BMI1 axis plays an important role in medulloblastoma progression (79). *BMI1* down-regulation leads to reduced protein kinase B (PKB) phosphorylation, impairing cell survival after chemotherapy (80). Thus, inappropriate signalling or defects in the Hh pathway can lead to cancer (77). Active Hh signalling can interfere with cancer treatment by inducing an aggressive or resistant tumor response to treatment (77). DNA methylation also plays an important role in regulating the Hh pathway. For example, hypomethylation of the Shh promoter causes over-expression of Shh ligands in breast as well as gastric cancer (81). This over-expression is due to the binding of NF-$_k$B, which initiates transcription of Shh (82). Thus, Hh signalling is upregulated in breast cancer cells, enabling them to continuously self-renew (82). Interestingly, *EZH2* is upregulated in prostate cancer, predicting recurrence and simultaneously activating the polycomb repressive complex (PRC1 and PRC2), which is

required for progression of cancer (83). Moreover, EZH2 can silence the p27 tumor suppressor gene through histone H3 lysine 27 methylation, facilitating pancreatic stem cell survival (84).

In summary, the clinical contribution of *EZH2* and other PcG genes in epigenetic modulation of CSCs is largely unknown, though *BMI1*'s role in promoting resistance to therapy has made progress. However, *BMI1*'s function could vary from one tissue to another (85).

1.1.6 DNA Methylation

The process of methylation occurs with the help of the enzyme DNA methyltransferase (DNMT) at the cytosine base that is generally adjacent to the guanine base of DNA, converting it to 5-methylcytosine (5-mC). This results in two methylated cytosine residues closer to each other on different strands of DNA (Fig. 1) (29, 86, 87). DNMTs also provide maintenance to CSCs by constantly replenishing epithelial cells, but the molecular pathway responsible is still unclear. It has been discovered that the expression of DNMT1 is much higher in mammary tumors (88)

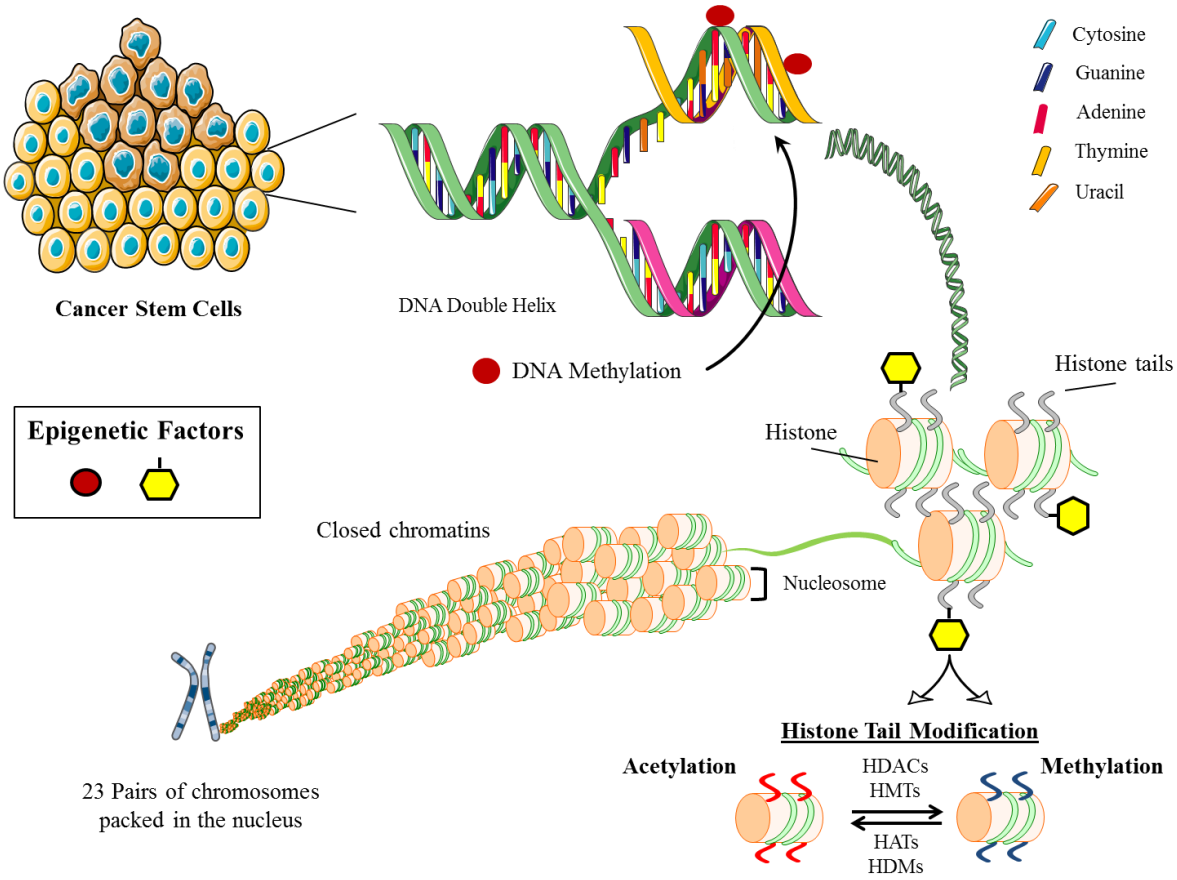

Figure 1: DNA methylation. Methyl marks are added to DNA bases to repress gene activity, influencing the way genes are expressed without changing the underlying DNA sequence. Furthermore, other epigenetics factors bind to histones modifying their tail. Histone tail modification controls when the chromatin complexes open up and allow their DNA to be read. (HAT: Histone acetyltransferase; HDAC: Histone deacetylase; HMT: Histone methyltransferases; HDM: Histone demethylases)

1.1.6.1 Demethylation in CSCs

The depletion of DNA methylation in genes is a very common characteristic of many types of cancers (89). One of the reasons for hypomethylation can be mutations in DNA methyltransferases, and its association with cancer has only been recently reported (89).

The process of removal of the methyl group from DNA is known as demethylation. It is an important process to overcome gene silencing or tumor progression. There are two ways by which DNA can be demethylated – passive and active. Passive DNA demethylation occurs in newly synthesized DNA with the help of DNMT1, whereas active demethylation occurs in DNA by the sequential modification of cytosine bases. It was also found that the addition of the demethylating agent 5-aza-2'-deoxycytidine can reverse the development of prostate CSCs by decreasing their stemness properties (90)

1.1.6.2 Hypermethylation in CSCs

Hypermethylation can occur in the promoter regions of tumor suppressor genes and lead to cancer. This process is being studied to use it as a treatment modality by reversing hypermethylation and ultimately terminate cancer. Hypermethylation of p*53* gene is necessary for the regulation of apoptosis in the cell (91-93).

In a healthy cell, apoptosis is triggered by any kind of change in the normal cell cycle or metabolic processes such as alteration in growth rate and intercellular signalling and exposure to radiation or toxic substances. The p53 gene is a well-known apoptosis regulatory gene (94). In a normal cell, *p53* is hypermethylated; however, a mutation can lead to deamination of 5-methyl cytosine, leading to the inactivation of *p53* and inhibiting apoptosis (95). A nuclear protein called mouse double minute 2 (MDM2) is known to repress the function of *p53*. However, MDM2 is obstructed by cyclin-dependent kinase inhibitor 4a gene (INK4a), which encodes for 2 proteins: p16INK4a and p14ARF. p14ARF is involved in suppression of MDM2, therefore allowing p53 to carry out apoptosis of the cell. Due to the mutation, hypermethylation of INK4a/ARF leads to inactivation of p14ARF, and thus *p53* is inhibited by MDM2, which causes abnormal behaviour in the cell (96). The two main pathways responsible for apoptosis in a cell under normal conditions are mitochondrial and caspase-mediated pathways. The mitochondrial pathway mainly causes apoptosis by providing hypoxic conditions to the cells with the help of apoptotic linked proteins belonging to the BCL-2 family. B-cell lymphoma-2 (BCL-2)/adenovirus E1B interacting

protein (BNIP3) is activated and localized to the mitochondria, resulting in hypoxic conditions for the cell (97). However, the CpG islands in the promoter region of BNIP3 are susceptible to hypermethylation, which can help the cancer cell escape apoptotic execution (98).

1.1.6.3 Hypomethylation in CSCs

Hypomethylation is almost always associated with hypermethylation in a genome but at a different sequence (99); however, it is less frequent than hypermethylation. Hypomethylation is said to increase the chances of mutation in a gene, and hence the probability of carcinogenesis due to hypomethylation is much higher (100). It is also involved in chromosomal instability that can either arise due to chromatin rearrangement or any other mutation leading to cancer (101). It has been studied vastly and is much more understood in relation to its role in carcinogenesis as compared to other processes.

The percentage of methylation throughout the chromosome is unevenly distributed, with CpG islands mainly found in the unmethylated region of the chromosome (102). In healthy tissue, around 3.5 to 4% of the cytosine has been found to be methylated under normal conditions (103, 104). Mostly, the satellite and interspersed repeat sequences of the chromosome are methylated; however, some unidentified regions of the DNA in human brain are also found to be methylated (102). Disruption or alteration of epigenetic regulation can cause hypermethylation at specific regions and hypomethylation of other sequences in the chromosome (105). Hypomethylation can occur in the methylated regions including the promoter of CpG islands, inactivated X chromosome, any repeated sequences, and intragenic sequences in the human chromosome (106).

It has been demonstrated that the frequency of hypomethylation in the genomes of cancer cells is very high compared to a healthy genome by determination of the levels of 5-methyl cytosine (107, 108). Studies undertaken in the past 20 years that focused on DNA methylation have reported that the progression of cancer is greatly dominated by hypomethylation along with multiple hypermethylation of CpG islands (109-111).

The global hypomethylation rate in colon carcinoma and adenocarcinoma is about 8-10% (108). The genes involved in colorectal carcinoma, including CDH3 (P-cadherin) and CD133, are found to be demethylated. Similarly, in colon cancer, *LINE-1* has been observed to be

hypomethylated (112). The CpG islands in the promoters (CpG Island methylator phenotype) are always found to be hypermethylated, which in turn represses further transcription of tumor suppressor genes (113). *CDKN2A/p16* is also methylated in colorectal cancer (114).

1.1.7 Histone modification in CSCs

Histones are highly alkaline (basic pH) proteins that package DNA into dense units (called nucleosomes) in the nucleus of eukaryotic cells (115). Arginine and lysine are the amino acids mostly contributing to the alkaline nature of histones (116). A covalent post-translational modification such as acetylation, methylation, and phosphorylation that can alter gene expression is known as histone modification (115). It can initiate various biological processes such as repair of damaged DNA and activation by uncoiling the chromatin, or deactivation by compacting chromatin to effect gene transcription (117).

Each histone is involved in a particular reaction and thus the quantitative analysis of histones can help in understanding the role of epigenetics in cancer (Fig. 1).

Histone H3 at lysine 27 has been found to be associated with adult stem cell and cancer cell differentiation (118). However, it is still not clear whether the histone H3K27me3 contributes to CSCs in any way (118). Thus, a study was performed on the ovarian CSC line – A2780, to detect the impact of H3K27me3 in CSCs with the use of an H3K27 demethylase inhibitor – GSKJ4, which concluded that it might have an inhibitory impact on CSCs (118).

The main co-factors that are involved in histone modification and epigenetic changes are histone acetyltransferases (HAT), histone methyltransferases (HMT), and histone deacetylaces (HDAC) (115). Histone acetylation is responsible for uncoiling of DNA (Fig. 1), accelerating transcription, and hindering DNA methylation; whereas, histone deacetylation leads to gene silencing (119, 120). Thus, histone modification controls various epigenetic processes and is suspected to be involved in tumor progression (121). Studies have shown that histone H3-K9 and DNA methylation regulate telomerase reverse transcriptase (hTET) expression that controls the binding of c-myc at the E-box 1 site of the hTET promoter (122).

1.1.7.1 Histone acetylation

Acetylation occurs at many levels in the nucleus, such as transcription activation, DNA repair, and cell cycle regulation. Histone acetyltransferase is mainly involved in transcription since the addition of an acetyl group causes denaturation in DNA, which can initiate

transcription. The process of acetylation in cells can be reversed with HDACs. Recently, it has been shown that HDAC inhibitors have the capability to induce differentiated cancer cells to gain stem-like properties and enter a quiescent state (123).

Similarly, H4K20 (H4K20me3) also plays an important role in altering the DNA methylation status. It has been shown that the loss of trimethylation of H4K20 and acetylation of H4K16 (H4K16Ac) is associated with DNA hypomethylation in various tumors (124). Histone modifications are also associated with increased recurrence risk in prostate cancer (125).

1.1.7.2 Histone methylation

Gene silencing with the help of PRC2, DNA methylation or the combination of both in CSCs suppresses their differentiation (126). PRC2 consists of enhancer of zeste homolog 2 (EZH2) that acts as a catalyst in the process of trimethylation of histone 3 lysine 27 (126). The PRCs interact with DNMTs to silence the tumor suppressor genes during cancer progression. In CSCs, the chromatins trigger the recruitment of DNMTs, leading to *ex novo* methylation and gene silencing. However, Histone H3 lysine 27 silences tumor suppressor genes in a DNMT-independent manner (127), showing the PcG targets extend beyond histone methylation. PcG target genes are more likely to be DNA hyper-methylated in cancers, as shown in colon and embryonic carcinoma cell lines (128-130), suggesting that EZH2 recruits DNA methyltransferase to the promotor region of PcG target genes and resulting in silencing of PcG targets and tumor suppressor genes (131). This indicates the cross-talk between DNA methylation and histone, resulting in a repressive effect. Furthermore, the combined epigenetic repressive effect was established in response to cancer cell DNA damage generated by reactive oxygen species (ROS) (132). Knowing this, it could be inferred that aberrant EZH2 activity, chromatin signatures, and ROS lock the adult somatic cells into an aberrant CSC state (133).

Given the importance of EZH2's role in increasing self-renewal in some hematological malignancies, therapies targeting EZH2 inhibition may promote CSC population inhibition (126, 134).

1.1.8 Role of chromatin remodelling in carcinogenesis

Recent studies show that genetic variations such as mutations, translocation or viral integration can cause inadequacy in epigenetic processes (135). This can also be explained

by Knudson's 'two-hit' hypothesis to cause malignancy in some cases (136). We know that processes such as molecular signalling or down-regulation and upregulation of genes are involved in cancer (137). Thus, studying epigenetics can help in finding the main cause of cancer and eliminate it in the future.

It has been proven that various types of mutations that can cause inactivation of *Dnmt1* are involved in the process of carcinogenesis in humans. In a recent study, somatic mutations in *Dnmt1* have been shown to be related to colorectal cancer; however, the main cause of cancer was still unclear (138). Chromatin remodelling can also alter cancer progression by inhibiting the process of methylation in genes (139).

1.1.9 Conclusions

Based on current evidence, we suggest models integrating signalling pathways (Wnt, Notch, and Hedgehog), epigenetic factors (DNA methylation and Histone modification), and chemo-resistance mechanisms (53). The understanding of cancer states and epigenetic abnormalities may play a key role in determining patterns in different CSC populations. We discussed the concept of molecular progression for epigenetic abnormalities before tumor initiation, which eventually modulates the epigenetic changes in CSCs. The role of epigenetics in CSC biology is very important considering that CSC plasticity is tightly linked to the peculiar epigenetic profiles, and PcG genes (BMI1 and Ezh2) are well established mediators of CSC epigenetic gene regulation.

Epigenetic alterations play a vital role in maintenance of stemness within the CSCs, enhancing the immortality of tumors. As different strategies have been employed to target bulk tumors, inhibitors of epigenetic modulatory enzymes such as DNMTs and HDACs, are potential targets for CSC inhibition. Already, older drugs targeting epigenetics, such as various demethylating agents and HDAC inhibitors are showing promise to sensitize tumors to other therapies (140). The tumor suppressor proteins play an important role in chromatin remodelling; however, restoration of normal chromatin remodelling by gene therapy is a potential therapeutic strategy to treat cancers with aberrant chromatin remodelling. We are convinced that epigenetic factors such as DNA and histone methylations/ acetylation and miRNAs are implicated in CSC epigenetic mechanisms. However, epigenetic therapy has the potential to lead epigenetic drugs to reverse and/or delay CSC resistance and can complement existing cancer therapies. Finally, epigenetic factors related to CSCs should be

considered as therapeutic targets to prevent and eradicate cancer. Future studies are required to identify PcG targets in CSCs and to link these pathways to CSCs chemo-resistance and epigenetic modulation.

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Author contributions

AbhiD drafted the outline and generated the figure. AbhiD wrote the manuscript. MB contributed to the manuscript. AD conceived of the study and AD, FA, and PN critically reviewed, revised, and approved the final manuscript.

Declaration of interests

The authors declare that they have no competing interests.

1.1.10 REFERENCES

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1.2 Cancer stem cell metabolism: A potential target for cancer therapy

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Abstract

Cancer Stem cells (CSCs) are a unipotent cell population present within the tumour cell mass. CSCs are known to be highly chemo-resistant, and in recent years, they have gained intense interest as key tumour initiating cells that may also play an integral role in tumour recurrence following chemotherapy. Cancer cells have the ability to alter their metabolism in order to fulfil bio-energetic and biosynthetic requirements. They are largely dependent on aerobic glycolysis for their energy production and also are associated with increased fatty acid synthesis and increased rates of glutamine utilisation. Emerging evidence has shown that therapeutic resistance to cancer treatment may arise due to dysregulation in glucose metabolism, fatty acid synthesis, and glutaminolysis. To propagate their lethal effects and maintain survival, tumour cells alter their metabolic requirements to ensure optimal nutrient use for their survival, evasion from host immune attack, and proliferation. It is now evident that cancer cells metabolise glutamine to grow rapidly because it provides the metabolic stimulus for required energy and precursors for synthesis of proteins, lipids, and nucleic acids. It can also regulate the activities of some of the signalling pathways that control the proliferation of cancer cells.

This review describes the key metabolic pathways required by CSCs to maintain a survival advantage and highlights how a combined approach of targeting cellular metabolism in conjunction with the use of chemotherapeutic drugs may provide a promising strategy to overcome therapeutic resistance and therefore aid in cancer therapy.

Keywords: cancer stem cells; metabolism; glycolysis; glutaminolysis; cancer therapy; chemo-resistance; tumour microenvironment; Wnt signalling

1.2.1 Introduction

Chemotherapy, along with radiotherapy and hormone therapy, is the most common treatment for cancer. Due to the side effects of treatment and chemo-resistance of the tumour cells, researchers have shifted their focus to more site-specific treatments in order to achieve better results (1).

Over the past decade, a critical role of a small subset of tumour cells, known as cancer stem cells (CSCs), was established in tumour relapse and propagation (2, 3). Most solid tumours, including breast, brain, prostate, ovary, mesothelioma, and colon cancer contain this small subset of self-renewing tumour initiating cells (4). Conventional anti-cancer therapies inhibit/kill the bulk of the heterogeneous tumour mass, resulting in tumour shrinkage. However, it has been suggested that later, the CSCs differentiate into tumour cells and are responsible for tumour relapse (Figure.1). The identification of novel therapies to target CSCs has been the goal of many cancer research laboratories, and recent studies suggest the CSCs undergo metabolic alterations that include low mitochondrial respiration and high glycolytic activity. Exploiting the CSCs' metabolic alterations may provide new effective therapies and diminish the risk of recurrence and metastasis (5, 6).

1.2.2 Cancer stem cells

The origin of CSCs is still unclear and further studies are required in each type of cancer. CSCs are known to remain in G0 phase, the resting phase of the cell cycle, and express high drug efflux transport systems. CSCs, being in a dormant state, make it difficult for most anti-cancer drugs that target only proliferative tumour cells. CSCs exhibit specific characteristics such as self-renewal and heterogeneous differentiation capacity, small population (0.001-0.1%) (7, 8), resistance to chemo/radiotherapy, high metastatic ability, sphere forming ability, and high ABC transporter expression. CSCs are also known to have a high migratory capacity, enabling their spread from the primary tumour to secondary sites. Various techniques have been established to isolate CSCs from the tumour mass and characterise them. CSCs are niche forming cells enriched with growth factors (such as epidermal growth factor and fibroblast growth factor). CSCs are characterised by specific surface markers such as $CD133^+/CXCR4^+$, $CD24^+/CD44^+$, $CD24^+/CD44^+/ESA^+$, $c\text{-}Met^+/CD44^+$, and $ALDH1^+/CD133^+$ in pancreatic cancer (9, 10); $CD24^{-/low}/CD44^+$ in breast cancer; $CD44^+$ in colon/ gastric/ head and neck/ovarian cancer; $CD34^+/CD38^-$ in leukaemia cells; CD13/CD45/CD90 in liver cancer;

CD117/CD90/EpCAM in lung cancer; CD20/CD166/Nestin in melanoma cancer; and $CD133^{+}/ABCG2^{+}$ in Glioblastoma Multiforme (11, 12). CSCs also express various markers such as CXCR4/ ESA and Nestin (9). CD44 is one of the most important CSC markers due to its capability to bind hyaluronic acid, which initiates CSC attachment to the extracellular matrix and contributes to tumour cell migration (13). The CSCs with $CD44^{+High}$ and $CD133^{+High}$ expression are highly radio-resistant in colon cancer, and they also have higher expression of AKT (AKT1/2) compared to $CD44^{Low}$ and $CD133^{Low}$ cells, indicating their capacity for higher DNA repair and the ability to escape cell death/apoptosis post radiotherapy (14). Therefore, selective targeting of these markers can be an effective way to deliver cytotoxic drugs to CSCs.

1.2.3 Targeting metabolic regulators

Understanding the mechanism by which CSCs are chemo-resistant and initiate tumour relapse is very important in order to address cancer therapy and to understand CSC biology (Figure. 1). B-cell lymphoma (Bcl-2) protein and its family members are known metabolic regulators, and it is recognised as a crucial mediator of mitochondrial apoptotic signalling. Its metabolic role was confirmed by the presence of Bcl-2 associated death promotor (15) in complex with glucokinase (16). Glucokinase has a low affinity for glucose transporter proteins and is purely substrate driven, making it an ideal substrate sensor to detect glucose in pancreatic Beta cells and hepatocytes (16). The activation of glucokinase is driven by phosphorylation of BAD by kinases such as Akt. The BAD`s pro-apoptotic capacity is inhibited when bound to glucokinase. However, dephosphorylated BAD, on dissociation with glucokinase, will bind to Bcl-2/xl, initiating apoptosis. Furthermore, it has been shown in some cancers that inhibition of BAD phosphorylation decreases cancer cell survival (17, 18).

The glucokinase complex and BAD accumulation will also promote glycolysis, which favours proliferation and CSC biosynthesis. However, dephosphorylation of BAD shifts the balance towards cell death and inhibits the metabolic signals necessary for high glucose flux to enable cell survival regulation (19).

The Bcl-2 protein family impairs the cell's ability to release apoptogenic protein cytochrome c from the mitochondria by mediation of the balance between cell survival and apoptosis. It achieves this by binding to the pro-apoptotic proteins Bcl-2 associated X protein (20) and

Bcl-2 homologous antagonist killer (BAK) (21). While the expression of Bcl-2 in CSC chemodrug resistance is still unclear, and it might be due to chromosomal translocation or other pathways, it was demonstrated that leukaemia $CD34^{+}$ cells expressed Bcl-2 and Bcl-X (22), and Bcl-2 was highly expressed in breast $CD44^{+}/CD24^{-/low}$ CSCs (23). To further understand the role of the Bcl-2 protein family, it was demonstrated that Bcl-2 expression in $CD133^{+}$ human hepatocellular carcinoma cells can be regulated by the Akt signalling pathways, where the Huh7 cancer cell line expressed higher levels of Bcl-2 in $CD133^{+}$ cells (24). Furthermore, Bcl-2 expression decreased when $CD133^{+}$ cells were treated with an Akt1 specific inhibitor, indicating the importance of Akt1 in the mechanism to mediate chemo-resistance in CSCs.

One of the mechanisms for cancer cells to achieve their metabolic shift is through modifications of metabolic and apoptotic roles of Bcl-2 family proteins. The metabolic alterations of this family of proteins may prove potent in increasing cancer cells' susceptibility to apoptosis and affecting tumorigenic metabolic reprogramming.

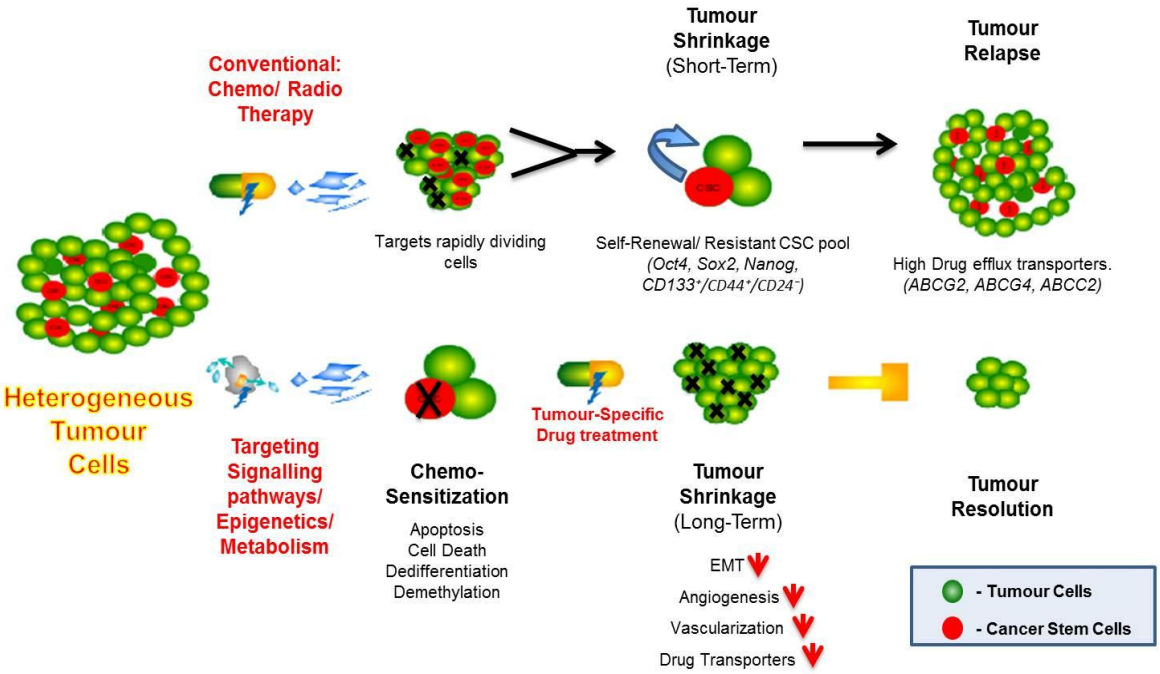

Figure 1: Cancer stem cell chemo-resistance. CSCs are a small sub-population of tumour cells that are highly chemo-resistant and play a prominent role in tumour relapse. The chemo-resistant property of CSCs is believed to contribute to poor prognosis of conventional tumour treatments. Therefore, we rationalise that therapy targeting CSCs would enable chemo-sensitisation by affecting downstream signalling pathways of tumour cells and enable the drugs to destroy the tumour bulk.

1.2.4 Targeting drug transporters

CSCs are known to possess a high efflux system that disables the chemo-therapeutic drugs' activity, resulting in the formation of highly drug-resistant tumours. CSCs have been found to express several Adenosine triphosphate–binding cassette (ABC) transporters such as ABCB1/P-gp/MDR1, ABCG2/ BCRP/MXR, and ABCB5. (25, 26). The ABC transporters are highly dependent on ATP generation in CSCs; thus, targeting CSC metabolism/glycolysis would lead to depleted ATP production and inhibition of ABC transporters. ABCG2 is considered as a high capacity transporter of various substrates including chemotherapeutic drugs (27). With this in mind, it is has been suggested that $ABCG2^{+}$ tumour cells can represent CSCs, which are known for their drug-resistance. Higher ABCG2 expression has been observed in various CSCs from lung (28), pancreas (29), and liver (30), and is co-expressed with CD133 in melanoma and pancreatic cancer cell lines (31, 32). It is suggested that ABCG2 expression is upregulated by hypoxia via hypoxia-inducible transcription factor complex HIF-1α and HIF-2α signalling (33).

ABCB1/ P-glycoprotein (P-gp) /MDR1 are known to be expressed in the majority of drug resistant tumours. Being a product of the multidrug resistance (MDR)1 gene, it acts as an ATP-dependent efflux pump to various anti-cancer drugs (34). CSCs derived from pancreatic tumour cells have higher expression of ABCB1 and ABCG2 (35). Furthermore, the ABCB1 inhibitors verapamil and PSC833 were unable to efficiently inhibit mitoxantrone efflux in leukaemic CSCs, showing that high expression of ABCB1 would lead to the development of chemo-resistant cells (36).

ABCB5β (a half-transporter) has been found in malignant melanoma and breast cancer, and is known to mediate doxorubicin resistance (37). The $ABCG5^{+}$ cells represent 2-20% of the melanoma tumours and have been shown to successfully recapitulate the tumour in immuno-deficient mice; however, these tumours were unable to regenerate $ABCG5^{+}$ cells, suggesting their limited stemness capacity (7).

Various MDR-reversing agents have been developed and have met with variable success in clinical trials. The first three generations of ABC inhibitors/modulators such as quinine, verapamil, cyclosporine-A, tariquitor, PSC 833, LY335979, and GF120918 are not advisable for clinical use due to their adverse cytotoxic effects (38). Inhibition of ABC transporters can also cause toxicity to a patient's normal stem cells, since these have an enhanced DNA repair mechanism, particularly bone marrow-derived stem cells. In addition, ABCG2 and

ABCB1 play a pivotal role in maintaining the blood brain barrier (39), and interfering with their normal function could have drastic consequences. Therefore, researchers are currently developing the fourth generation of ABC inhibitors, which are naturally occurring active compounds, in order to provide more effective therapies (40).

1.2.5 Targeting the tumour microenvironment

Tumour progression is due to adaptive cellular responses such as dormancy, invasiveness, and chemo-resistance in the tumour metabolic microenvironment (41). Adaptive behaviour of CSCs in this heterogeneous microenvironment is one of the characteristics of CSCs (42). The tumour microenvironment plays a pivotal role in cancer cell progression, particularly for CSCs, and it mostly involves hypoxia, nutrition, and low pH (43).

Hypoxia in the tumour microenvironment allows pro-angiogenic factors to stimulate new vessel growth within the solid tumour, although the vessels tend to be immature and exhibit poor perfusion (44). Hypoxia, due to its spatial and temporal heterogeneity in tumours, is difficult to treat (45). The hypoxic response within the microenvironment is regulated by Hypoxia inducible transcription factors, HIF-1α/HIF-2α. The migration, glycolytic, angiogenic, and cell survival pathways constitute the transcription targets of HIF1α (46). Hence, targeting HIF1α is a potential therapy for cancer treatment.

In hypoxic stress, the endoplasmic reticulum (ER) is inhibited, activating the unfolder protein response (UPR) (47). The UPR maintains ER homeostasis and its disruption initiates apoptosis. Aberrant activation by the UPR in the absence of the two ER membrane proteins PERK (PKR-like ER kinase) and IRE-1 (inositol-requiring) results in increased hypoxia and reduced growth rates (48, 49). The UPR is an important cellular response mechanism in cancer, playing a role in calcium homeostasis, redox status, and glucose deprivation within the tumour.

Another potential target within the microenvironment is mammalian target of rapamycin (mTOR). During cell stress, nutrient and energy depletion within the solid tumour, mTOR activates the signalling cascades responsible for metabolism and cell survival mechanisms (50, 51). The anti-diabetic drug metformin has exhibited potential anti-tumour activity; it reduces blood glucose levels, thereby inhibiting gluconeogenesis, and initiates AMPK (AMP-activated protein kinase) activation (52). AMPK regulates the mTOR activity through activation of the tuberous sclerosis protein 1 complex (TSC1/2) (53).

The microenvironment of tumours is more acidic (pH 6.5-6.9) compared to normal tissues (pH 7.2-7.5), resulting in tumours having poor vascular perfusion and increased glycolytic flux (54), (55). Knowing that tumour invasiveness is more active in an acidic microenvironment (56, 57), manipulating the tumour microenvironment pH by orally distributed systemic buffers is an effective way to increase the extracellular pH of tumours (58), (59).

1.2.6 Targeting glycolytic enzymes to reduce chemo-resistance in CSCs

Most cells satisfy their energy demands through glucose catabolism, which is subject to complex regulation. To inhibit glucose catabolism through the central pathway of glycolysis, various glycolytic enzymes or transporters must be targeted such as GLUT 1 - 4 (60), hexokinase (61), pyruvate kinase M2, and lactate dehydrogenase A (62).

Cancer cells have the ability to alter their metabolism in order to fulfil bioenergetic and biosynthetic requirements. The extracellular environment can be acidified by what is known as the 'Warburg effect' (a term referring to high levels of glycolytic pathway flux, even under aerobic conditions). When HIF-1α induces the expression of carbonic anhydrases, and there is an interaction with extracellular acidification, the pH ratio between the intracellular and extracellular environment is altered (63-66) . The resultant pH shift affects drug absorption within the cell. At the same time, glycolytic adenosine triphosphate (ATP) production and the transporter induced over-expression of HIF-1α contribute to a decrease in the cytoplasmic retention of anti-cancer agents (67, 68) .

1.2.7 Targeting tumour cell metabolism

To induce their lethal effects and maintain survival, tumour cells alter their metabolism to ensure survival, evade host immune attack, and proliferate (69). This clever strategy of tumour cells was exposed by Otto Warburg in the 1920s when he proved that, in spite of the presence of abundant oxygen, tumour cells metabolise glucose via glycolysis to produce lactate. They adopt this pathway in order to produce ATP through a fermentation process that is much faster compared to the conventional oxidative phosphorylation (respiration), and also avoids the requirement for mitochondrial oxidative phosphorylation. This meets the requirement for the tricarboxylic acid (TCA) cycle activity to be directed towards

biosynthesis rather than ATP production. Inner regions of tumours are known to be hypoxic (60, 69-71). However, the application of anaerobic glycolysis for energy supply is just one part of the metabolic transformation of tumour cells. In order to multiply and survive, the cell must be able to replicate its genome, protein and lipid content, and other important constituents, and also pass on important biomolecules to daughter cells. To accomplish this, the tumour cells enhance the expression of glucose transporters (GLUTs) and monocarboxylate transporters (lactate/pyruvate) to ensure that glucose is delivered and that lactate is transported out of the cell (69, 72) (Figure 2). Glutamine (via glutamate) and some of the pyruvate enters the TCA cycle to initiate the precursor supply towards biosynthetic reactions. The theoretical significance of the Warburg effect can be illustrated by the glucose uptake and solvent capacity of the cell cytoplasm, i.e. the maximum number of macromolecules that can be accommodated in the intracellular space. Thus, when the glucose uptake rate is low, glucose uptake capacity is the limiting factor and mitochondrial respiration becomes the preferred source for ATP generation. At a high glucose uptake rate, the cell identifies the solvent capacity as its prime source for generating ATP, which in turn activates aerobic glycolysis and lessens mitochondrial respiration (Figure 2). Hence, the Warburg effect is the amicable catabolic choice for proliferating tumour cells (73). The other interesting outcome elicited by the Warburg effect is the creation of a tumour environment that facilitates survival and proliferation of the tumours. In the process of their expansion, the tumours stretch the diffusion limits of their oxygenated blood supply and thus induce hypoxia and stabilize the transcription factor HIF. HIF triggers angiogenesis by regulating various associated factors, especially vascular endothelial growth factor (74, 75). The other strategy adopted by these tumour cells to maximize their survival and proliferation is to increase their glutamine use for supply of biosynthetic precursors. Glutamine acts as a source of carbon and nitrogen for biosynthetic reactions of cancer cells. It gets converted to glutamate, enters into the TCA cycle, and acts as a precursor for the synthesis of important intermediates such as NADPH, anti-oxidants and amino acids such as α-ketoglutarate, aspartate, glutathione, and nucleic acids. The glutamine is converted to glutamate by the mitochondrial enzyme glutaminase. Glutaminase is highly expressed in rapidly growing tumour cells. Another link between oncogenic activation and tumour cell metabolism was determined when a study established that *c-Myc* increased glutaminase expression by suppressing miR-23a/b (69, 76, 77). Glutamine may be partially or fully oxidised by tumour

cells (78). It acts as an energy source through catabolism or as a building block via anabolism in the body.

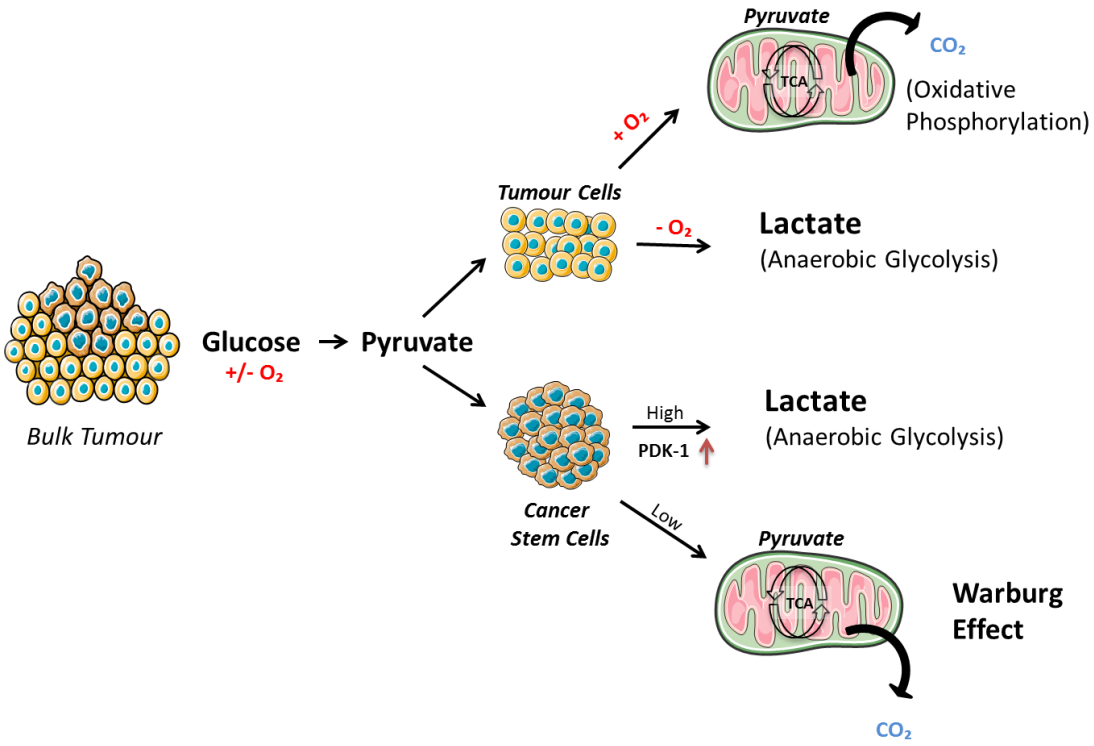

Figure 2: Impact of glucose utilisation by CSCs and non CSCs highlights the difference in their metabolic profiles. Pyruvate enters the TCA cycle to initiate the precursor supply towards biosynthetic reactions. The Warburg effect in turn activates aerobic glycolysis and lessens mitochondrial respiration, suggesting a preferred choice for proliferation.

1.2.8 Glutaminolysis in cancer metabolism

Cancer cells metabolise glutamine, as well as glucose, to grow rapidly because it provides the required ATP and essential biomolecules such as proteins, lipids, and nucleic acids (79). Glutamine influences the signalling pathways required for cancer cell proliferation, survival, and metabolism through regulation of mitochondrial reactive oxygen species (ROS) production (80, 81). Activation of the PI3-Kinase-Akt pathway results in increased production of ROS in mitochondria through metabolic pathways (82). Glutamine is first converted to glutamate by the enzyme glutaminase, and then glutamate is converted to α-ketoglutarate (αKG) by the action of glutamate dehydrogenase (or an aminotransferase). The rapidly growing tumour cells use glutamine as a carbon source for energy production and for the replenishment of TCA cycle intermediates such as pyruvate, oxaloacetate, and αKG to make up for the constant loss of citrate, which is exported out of the mitochondria for lipid synthesis. It has been observed that glutamine withdrawal in cells with increased *c-Myc* expression led to the death of the oncogenic cells (77). Thus, it can be confirmed that cancer cells employ glutamine to provide substrates for the TCA cycle (80, 83). Further studies have also demonstrated that the oncogene *c-Myc* impacts glutamine metabolism, thus stimulating the glutamine transporters SLC5A1 and SLC7A1 and, as a result, promoting the expression of glutaminase 1 by suppressing the expression of miR-23A and miR-23B (84).

These data provide a concrete platform to include glutamine metabolism in cancer as an integral part of cancer therapeutic strategies. Glutamine analogues such as 6-diazo-5-oxo-L-norleucine (L-DON), acivicin, and azaserine were found to demonstrate anti-cancer activities but were not formulated into drugs due to their neuro- and gastrotoxicity (85). However, it has been shown that inhibition of glutamine metabolism via L-DON was able to reduce cancer metastasis in a VM-M3 mouse model (84, 86). Zhou et al (87) performed a proteomic analysis in pancreatic ductal adenocarcinoma that revealed the role of glutamine metabolism in cancer. They found that the level of glutaminase in the cancer cells was much higher compared to the normal ductal cells. In addition, the concentration of other enzymes such as cytidine triphosphate synthase, guanine monophosphate synthetase, and asparagine synthetase, which use glutamine as substrates, was found to be elevated in pancreatic cancer. This indicates that the high utilisation rates of glutamine by cancer cells

are required to satisfy their need of nitrogen and energy for uninterrupted, fast growth. Paediatric acute leukaemia has been successfully treated by L-asparaginase, which catalyses the hydrolysis of asparagine to aspartic acid. This enzyme is also capable of hydrolysing glutamine to glutamic acid and ammonia, thus reducing blood glutamine levels (84).

Histone deacetylase (HDAC) inhibitors such as phenylbutyrate have been used pharmacologically to inhibit the invasive properties of breast and prostate cancer by inducing apoptosis and depleting the blood glutamine levels (88, 89). It is generally used to treat hyperammonemia in urea cycle disorders, but it also brings down the level of glutamine in the plasma by forming a conjugate and thus helps curb tumour growth (90). The glutamine transporters SLC1A5 (ASCT2) and SLC1A7, which are over-expressed in various human cancers such as colon, liver, colorectal adenocarcinomas, glioblastoma multiforme, and melanoma, have been attractive targets due to their role in cell survival signalling and also being a major source of glutamine delivery (91). IL-γ-glutamyl-p-nitroanilide has been shown to inhibit SLC1A5 (ASCT2) and cause autophagy in cancer cells (83, 84). A chemical compound termed 968 exhibited anti-glutaminase activity, which in turn suppressed the oncogenic transformation by *c-Myc* via down regulation of miR-23, which has been seen in prostate cancer and human B-cell lymphoma (84). Another compound, Bis-2-(5-phenylacetamido-1, 2, 4-thiadiazol-2-yl) ethyl sulphide, also exhibited inhibitory effects on glutaminase, thus repressing glutamine availability to the cancer cells (85). Ongoing and future work would aim at presenting a more detailed picture of glutamine metabolism and its involvement in cancer, which would help develop safe and effective glutamine inhibitors.

1.2.9 CSCs and their metabolic alterations

Although much is known regarding metabolic pathways important for tumour survival, the potential for therapeutic metabolic alteration of CSCs still remains under investigation (92), (93). Recent studies indicate that CSCs have different metabolic properties when compared to the tumour bulk. One such study on brain tumour CSCs revealed that these cells show a low activity of mitochondrial respiration (68). This finding triggered the need to study the effect of glucose in the microenvironment of CSCs because glucose was estimated to be critical for the CSCs. It has been found that CSCs have higher glycolytic rates than other tumour cells (94). Glucose induced the expression of specific genes in CSCs associated with

glucose metabolism and the Akt pathway (*c-Myc*, *GLUT1*, *HK-1*, *HK-2*, and *PDK-1*), which contributes to the rise in the CSC population (94). Glucose utilisation by CSCs and non CSCs was compared by measuring their glucose consumption and lactate production rates in order to establish evidence for the difference in metabolic profiles of CSCs and the bulk of the tumour. It has been observed that glucose uptake, lactate production, and ATP content are elevated in CSCs as compared to the non CSCs (Figure 2) (84, 95, 96). Many crucial molecules involved in glucose metabolism have been studied in relation to the metabolism of CSCs, such as hexokinase-1 (HK1), hexokinase-2 (HK2), pyruvate dehydrogenase kinase 1 (PDK1), and glyceraldehyde-3-phosphate dehydrogenase. The PDK1 levels are high in the CSC population (Figure 2). PDK-1, via the TCA cycle, phosphorylates pyruvate dehydrogenase and suppresses the pyruvate to acetyl-CoA conversion. Furthermore, suppressing the metabolic flow of pyruvate in mitochondria induces the conversion of pyruvate to lactate in the cytosol (94, 97). HK-1 and HK-2 both catalyse the conversion of glucose to glucose-6-phosphate in glycolysis, but the levels of HK-2 are lower in CSCs while that of HK-1 are higher, suggesting that HK-1 maintains CSCs' glycolytic activity. Interestingly, *HIF-1α* and *c-Myc* expression (affects HK-2 expression) didn't change in CSCs and tumour cells. The increase in expression of proteins in the Akt signalling pathway bestows CSCs with a longer life span (70, 94).

Palorini et al (96) studied the effect of glycolysis inhibition and glucose deprivation on the CSC cell line 3AB-OS, which was derived from the human osteosarcoma cell line MG63. They have reported that the 3AB-OS cells require glucose for survival and proliferation. The absence of glucose caused death of the CSC cell line. Glutamine deprivation led to a decline in the MG63 population, which suggested that the 3AB-OS population was not greatly affected by withdrawing glutamine.

Hence, incorporating these features into therapeutic strategies to treat cancer can produce an extensively efficient treatment for various cancers. Also, combining glycolytic inhibition strategies with existing chemotherapy can also help eliminate tumour load completely because the CSCs will also be targeted (96).

1.2.10 Conclusion

The targeting of CSCs is emerging as a novel therapy to eradicate the progression of various cancers. The inefficiency of traditional anti-cancer therapies lay the stepping stone for studying the metabolism of cancer cells and the pathways controlling and regulating their growth and proliferation, and converting them into formidable treatment options. Targeting the special metabolic traits of CSCs would enable the basis for the development of new therapeutic strategies to inhibit the bulk of the tumour. Clinically, targeting the CSCs resistant towards therapy and metastasis would enable long term disease free survival for the patients.

Though, drug development for CSC metabolism is gaining wide interest, it is still controversial issue as there are studies contradicting the glycolytic phenotype of CSC and oxidative state of CSCs.

On the other hand, cancer cell metabolism has emerged to be one of the most fascinating and promising areas in cancer therapy research. The current research focuses on trying to understand the metabolic demands and profile of cancer cells, and design drugs accordingly in order to add a new exciting chapter to cancer treatment. Also, drugs targeting cancer metabolism can be employed for multiple cancers, which can possess a broad spectrum of activity, and are indeed under clinical trials that will likely result in new treatment options in the future (98). Despite the limited information on CSC metabolic activity and their ability to self-renew, tumour initiation, differentiation capacity, chemo-resistance and survival strategy, targeting CSC metabolism holds great promise in translating into effective cancer treatment. However, combinatorial treatments involving both standard chemotherapeutic drugs and chemo-sensitizing agents on CSCs would probably be the most efficient CSC-targeted therapy (Figure 1).

Abbreviations

CSCs: Cancer stem cells; Bcl-2: B-cell lymphoma; ABC: ATP binding cassette; SDF1: stromal cell derived factor 1; PTCH1: patched homologue; PDK1: pyruvate dehydrogenase kinase 1; HK: hexokinase; TCA: tricarboxylic acid cycle; αKG: α-ketoglutarate.

Competing interests

The authors declare that they have no competing interests.

Authors' contributions

Abhijeet D drafted the outline and generated the figures. Abhijeet D wrote the manuscript. KD contributed to the manuscript. AD conceived of the study and AD, FA, and PN critically reviewed, revised, and approved the final manuscript.

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Chapter 2

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2 Secreted Frizzled-related protein 4 (sFRP4) chemo-sensitizes cancer stem cells derived from human breast, prostate, and ovary tumor cell lines

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Abstract

This study investigated molecular signals essential to sustain cancer stem cells (CSCs) and assessed their activity in the presence of secreted frizzled-related protein 4 (sFRP4) alone or in combination with chemotherapeutic drugs. SFRP4 is a known Wnt antagonist, and is also pro-apoptotic and anti-angiogenic. Additionally, sFRP4 has been demonstrated to confer chemo-sensitization and improve chemotherapeutic efficacy. CSCs were isolated from breast, prostate, and ovary tumor cell lines, and characterized using tumor-specific markers such as $CD44^{+}/CD24^{-}/CD133^{+}$. The post-transcription data from CSCs that have undergone combinatorial treatment with sFRP4 and chemotherapeutic drugs suggest downregulation of drug transporters and upregulation of apoptotic and cell death markers. The post-translational modification of CSCs demonstrated a chemo-sensitization effect of sFRP4 when used in combination with tumor-specific drugs. SFRP4 in combination with Doxorubicin/Cisplatin reduced the proliferative capacity of the CSC population *in vitro*. Wnt/β-catenin signalling is important for proliferation and self-renewal of CSCs in association with human tumorigenesis. The silencing of this signalling pathway by the application of sFRP4 suggests potential for improved *in vivo* chemo-responses.

2.1 Introduction

Chemotherapy, along with radiotherapy and hormone therapy, is the most common treatment for cancer. Due to the side effects of treatment and chemo-resistance of tumor cells, researchers have shifted their focus to more site-specific treatments in order to achieve better patient outcomes (1). Over the past decade, a critical role of a small subset of tumor cells, known as cancer stem cells (CSCs), was established in tumor relapse and propagation (2, 3). Most solid tumors, including breast, brain, prostate, ovary, mesothelioma, and colon cancer contain this small subset of self-renewing, tumor initiating cells (4). Conventional anti-cancer therapies inhibit/kill the bulk of the heterogeneous tumor mass, resulting in tumor shrinkage. However, it has been suggested that later, the CSCs differentiate into tumor cells and are responsible for tumor relapse (5, 6). CSCs are characterized by their tumor forming ability and expression of high levels of ATP-binding cassette drug transporters (ABCG2), cell adhesion molecules (CD44), and anchorage independent cell survival proteins (Cyclin D1), which are collectively responsible for chemo-resistance (7-9). In human breast, ovary, and prostate cancers, several CSC populations have been identified using cell surface markers ($CD44^+/CD133^+/ CD24^{-/low}$); these CSCs have shown a high clonal, invasive, and metastatic capacity, leading to resistance to radio-therapy, chemotherapeutic drugs (Doxorubicin and Cisplatin), and other target-specific therapy (10-12).

CSCs possess high capacity for tumor propagation and metastasis (13-15), which causes more than 90% of cancer-related deaths. The molecular mechanism of CSCs regulating metastasis is not completely understood; however, the invasive metastatic cascade involves circulation of cancer cells through the surrounding extracellular matrix in a multistep cellular operation.

The development and maintenance of CSCs is controlled by several signalling pathways such as Wnt and Notch. The Wnt pathway is known to mediate the self-renewal capacity of CSCs through modulation of β-catenin/TCF transcription factors. There is evidence suggesting a Wnt signalling role in CSC maintenance (as seen in murine models and humans) of non-

melanoma cutaneous tumor, where CSCs are maintained by Wnt/β-catenin signalling (16). The interactions of Wnt proteins to the receptor complex can be inhibited by binding of the ligands to endogenous Wnt antagonists such as secreted frizzled-related proteins (sFRPs) (17). SFRP4 is one of the prominent isoforms with the capacity to chemo-sensitize tumor cells to chemotherapeutics (18, 19). Chemo-sensitization of CSCs by sFRP4 has the potential to decrease the required chemotherapeutic load to facilitate tumor resolution.

2.2 Results

2.2.1 Tumor derived CSCs characterization

Spheroids obtained for CSC isolation were characterized for the expression of tumor-specific CSC markers $CD44^+$ $CD24^{-/low}$ for breast CSCs, and $CD133^+$/ $CD44^+$ for prostate and ovarian CSCs (Table 1), by using flow cytometry. The combinatorial treatment showed significant reduction in the CSC marker population in all cell line-derived CSCs; although in A2780 prostate CSCs, Cisplatin treatment showed phenotype switching to $CD44^+$ positive cells and only reduced the $CD133^+$ population; however, this switching did not affect the inhibitory effect of combinatorial treatment (see Supplementary Figure 1). The characterized CSCs were further used for functional analysis.

Treatment	Surface markers	$CD44^+/ CD24^-$ (%)		$CD133^+/ CD44^+$ (%)				
		MDA231	MCF 7	PC3	LnCa p	A2780 P	A2780 ADR	A2780 CIS
Untreated		58.1	36.7	24.3	62.7	2.85	19.5	2.72
sFRP4		32.3	17.2	17.2	44.1	2.99	16.8	2.21
Dox./ Cisplatin		28.5	14.9	22.1	47.5	9.29	16	1.8
sFRP4 + Dox./ Cisplatin		**25**	**1.68**	**9.16**	**10.4**	**1.88**	**15.1**	**1.23**

Table 1: Effect of sFRP4 on CSC surface markers: Using flow cytometry, statistical analysis of CSC markers was performed post treatment. Data are means ± percentage from 3 independent experiments.

2.2.2 SFRP4 in combination with Doxorubicin/Cisplatin reduces the sphere forming capacity of CSCs.

The CSCs derived from breast, prostate, and ovary tumor cell lines were treated with sFRP4 (250 pg/ml) and Doxorubicin (5µM)/Cisplatin (30µM) alone or in combination. The untreated spheroids remained intact, whereas the combinatorial treatment of sFPR4 and chemotherapeutic drugs showed disruption of spheroids post-treatment (Figure 1), indicating sFRP4's capacity to segregate the tumor spheres and allow chemotherapeutic drugs to inhibit tumor proliferation. This was further confirmed by immunofluorescence, where spheroids were labelled with $CD44^{+}/CD24^{-/low}$/ ABCG2/ KI67 for CSCs derived from breast tumors (Figure 2a and 2b), and $CD133^{+}$/ $CD44^{+}$/ ABCG2/ Ki67 for prostate (Figure 2c and 2d) and ovarian tumors (Figure 2e, 2f and 2g); except for the prostate LnCap CSCs, which were not $CD44^{+}$ (Figure 2d). The combinatorial treatment showed sphere disruption and a reduction in surface receptor expression compared to sFRP4 and drugs alone, indicating the effect of sFRP4 in inhibiting the spheroids' proliferative capacity.

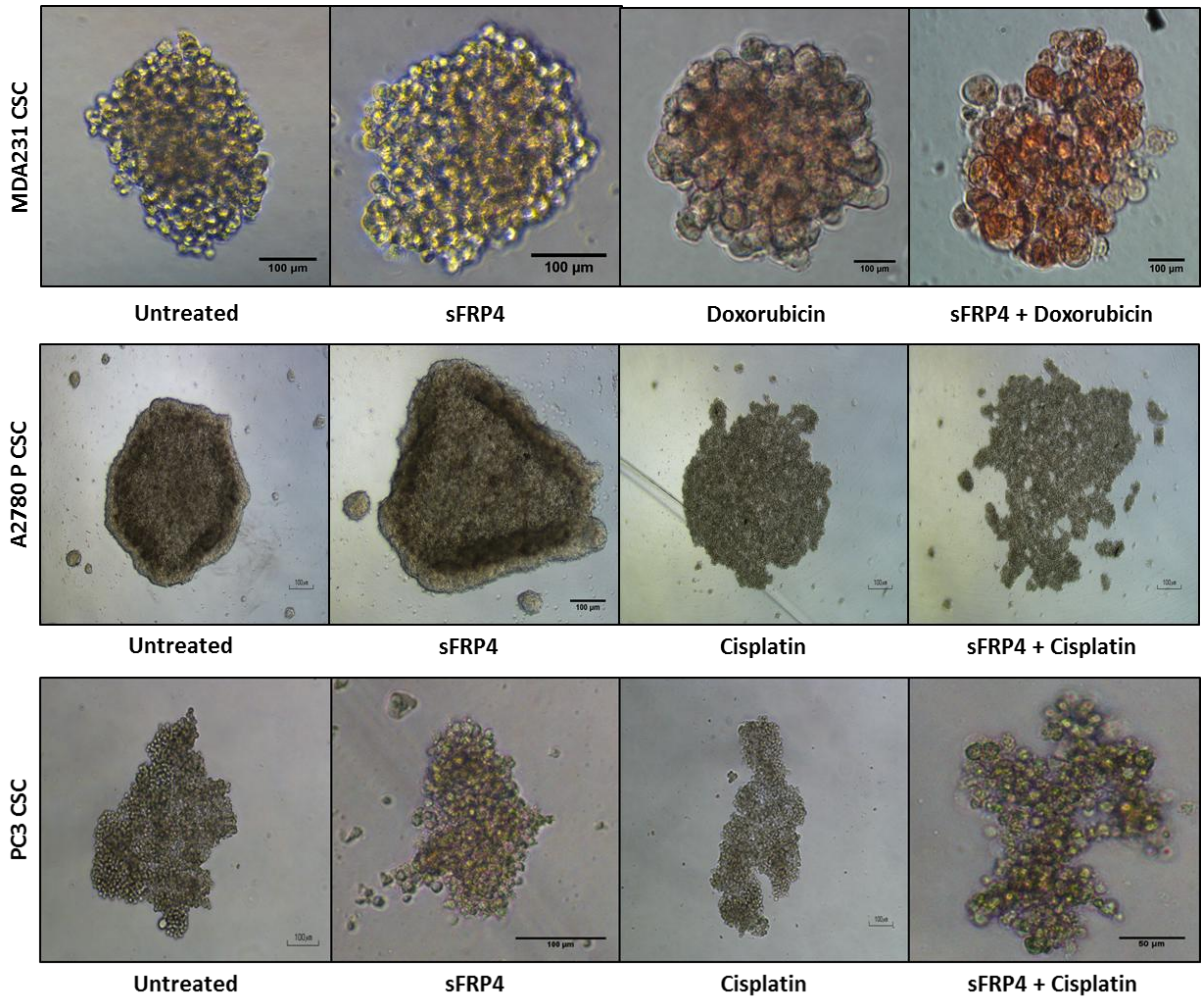
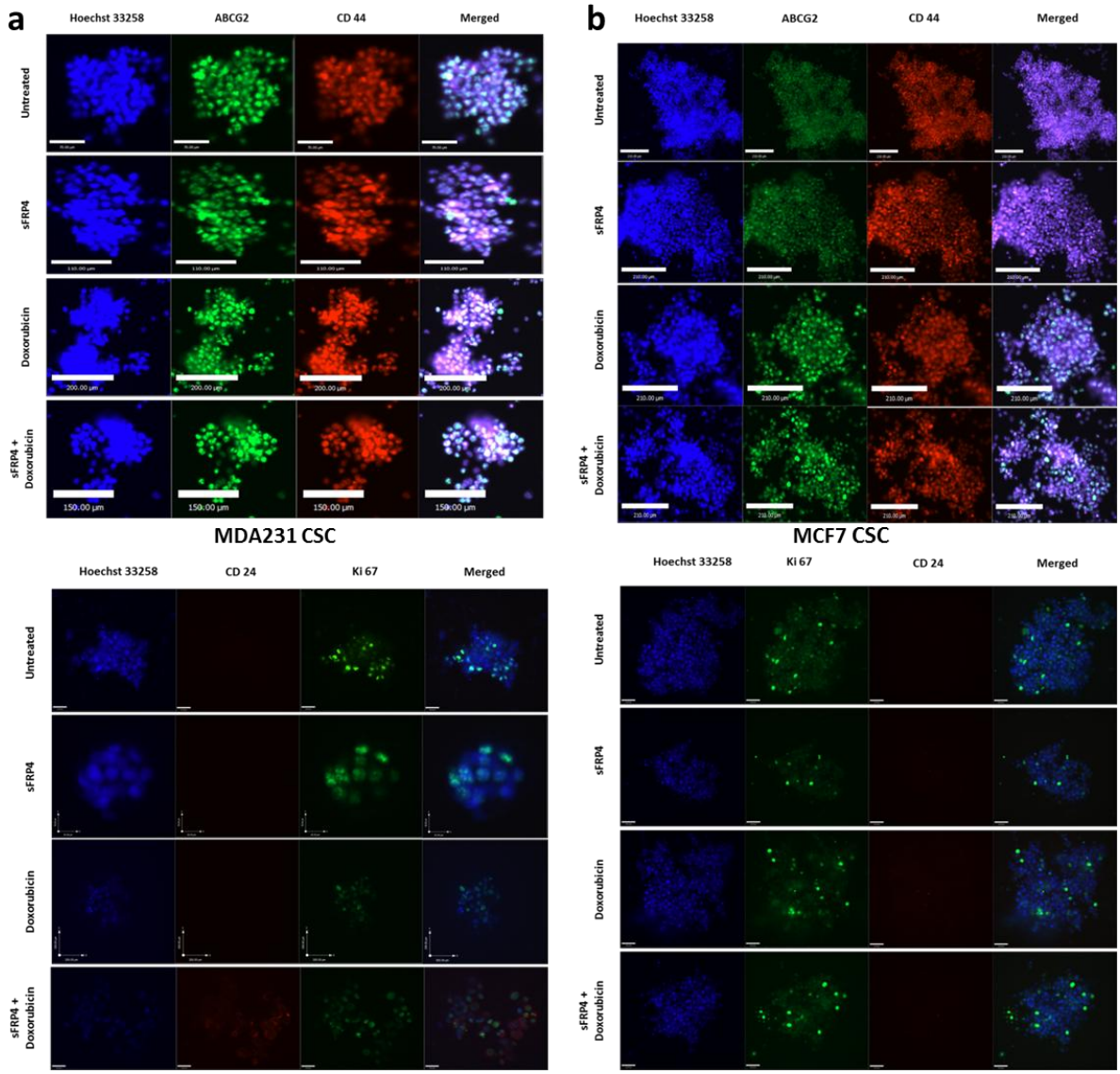
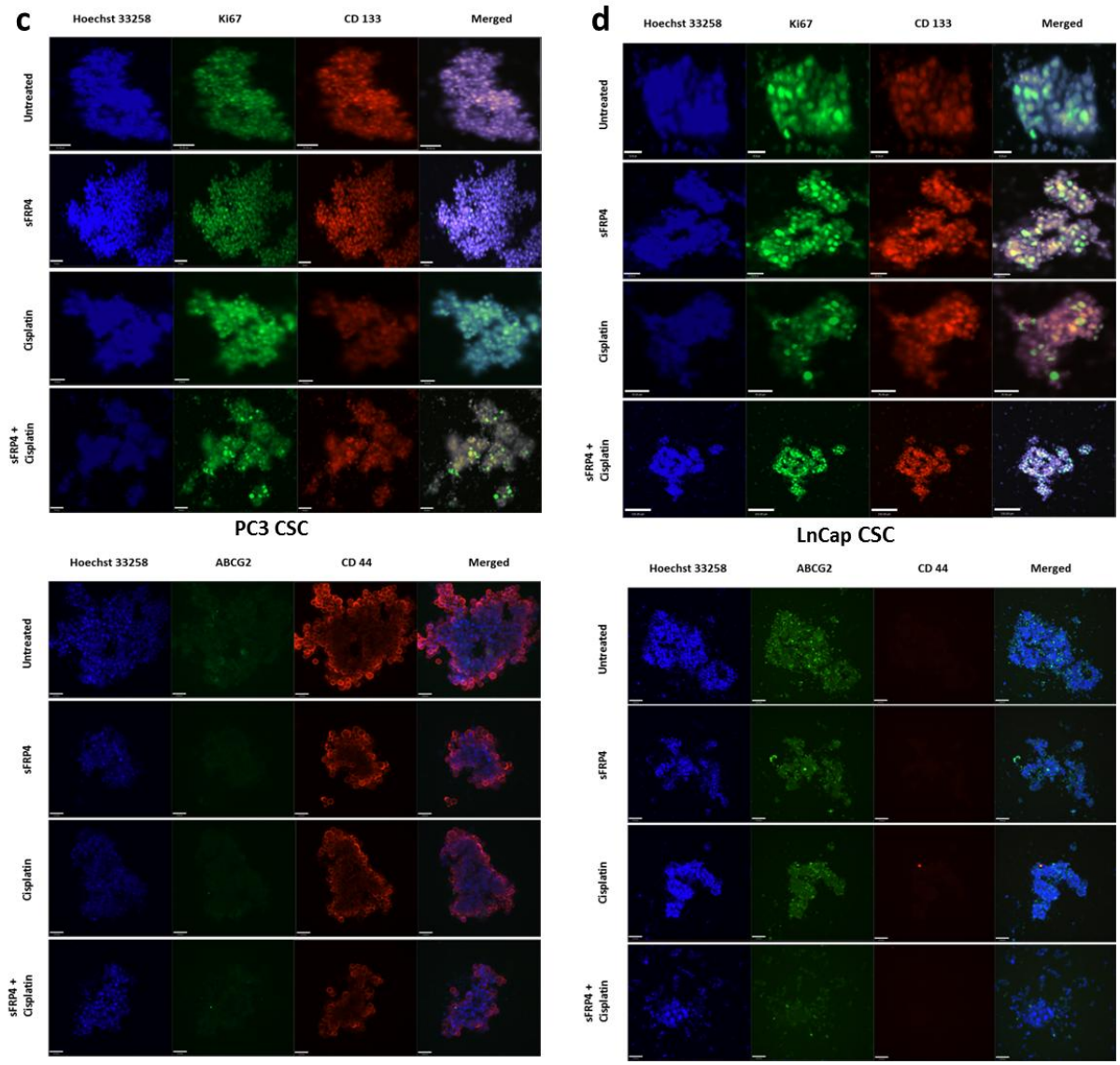


Figure 1: Effect of sFRP4 on CSC Morphology: CSCs were isolated from breast, ovary, and prostate tumor cell lines and treated with sFRP4 (250pg/ml) with chemotherapeutic drugs (Doxorubicin 5µM/Cisplatin 30µM). The combinatorial treatment shows the disruption of the CSC sphere. (Scale bar: 100 µm). Cells images are representative from all the experiments


a
Hoechst 33258
ABCG2
CD 44
Merged
Untreated
sFRP4
Doxorubicin
sFRP4 + Doxorubicin
150.00 µm
MDA231 CSC
b
Hoechst 33258
ABCG2
CD 44
Merged
Untreated
sFRP4
Doxorubicin
sFRP4 + Doxorubicin
MCF7 CSC
Hoechst 33258
CD 24
Ki 67
Merged
Untreated
sFRP4
Doxorubicin
sFRP4 + Doxorubicin
Hoechst 33258
Ki 67
CD 24
Merged
Untreated
sFRP4
Doxorubicin
sFRP4 + Doxorubicin


c
Hoechst 33258
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CD 133
Merged
Untreated
sFRP4
Cisplatin
sFRP4 + Cisplatin
PC3 CSC
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ABCG2
CD 44
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sFRP4
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sFRP4 + Cisplatin
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sFRP4 + Cisplatin
LnCap CSC
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ABCG2
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Merged
Untreated
sFRP4
Cisplatin
sFRP4 + Cisplatin

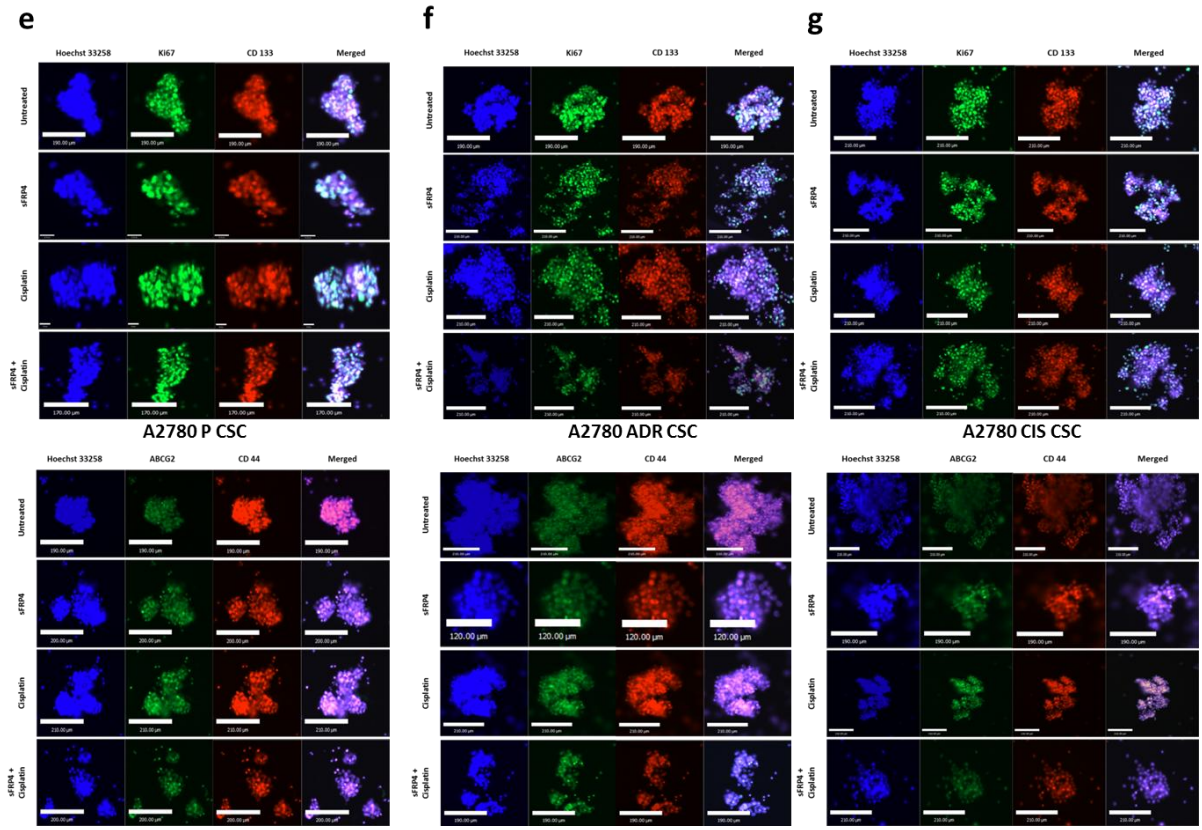


Figure 2: Effect of sFRP4 on CSC cell surface markers: Using Immunofluorescence, $CD44^{+}$/ $CD24^{-}$/ $CD133^{+}$/ Ki67/ ABCG2 were detected in all the tumor cell line-derived CSCs. sFRP4 in combination with Doxorubicin/Cisplatin showed disruption of the spheres. Nuclei were counterstained with Hoechst 33342 (blue). a/b) $CD44^{+}$/ $CD24^{-}$ breast tumor cell line CSCs (MDA231/MCF7). c/d) Prostate tumor cell line derived CSCs. PC3 expressed $CD44^{+}$/ $CD133^{+}$/ Ki67/ ABCG2. LnCap was negative for $CD44^{+}$. e/f/g) $CD44^{+}$/ $CD133^{+}$/ Ki67/ ABCG2 observed in ovary tumor cell line-derived CSCs (A2780 P/ CIS/ ADR). Immunofluorescence images are representative from 3 independent experiments. (Scale bar: 100 µm)

2.2.3 SFRP4 in combination with Doxorubicin/Cisplatin reduces CSC viability

Using an MTT assay, it was observed that the combinatorial treatment of sFRP4 and Doxorubicin/Cisplatin significantly inhibited the viability of CSCs ($P<0.001$, $n=3$) compared to sFRP4 or drugs alone. Similar patterns were observed in all the cell lines (Figure 3). Therefore, this treatment combination was used for subsequent studies.

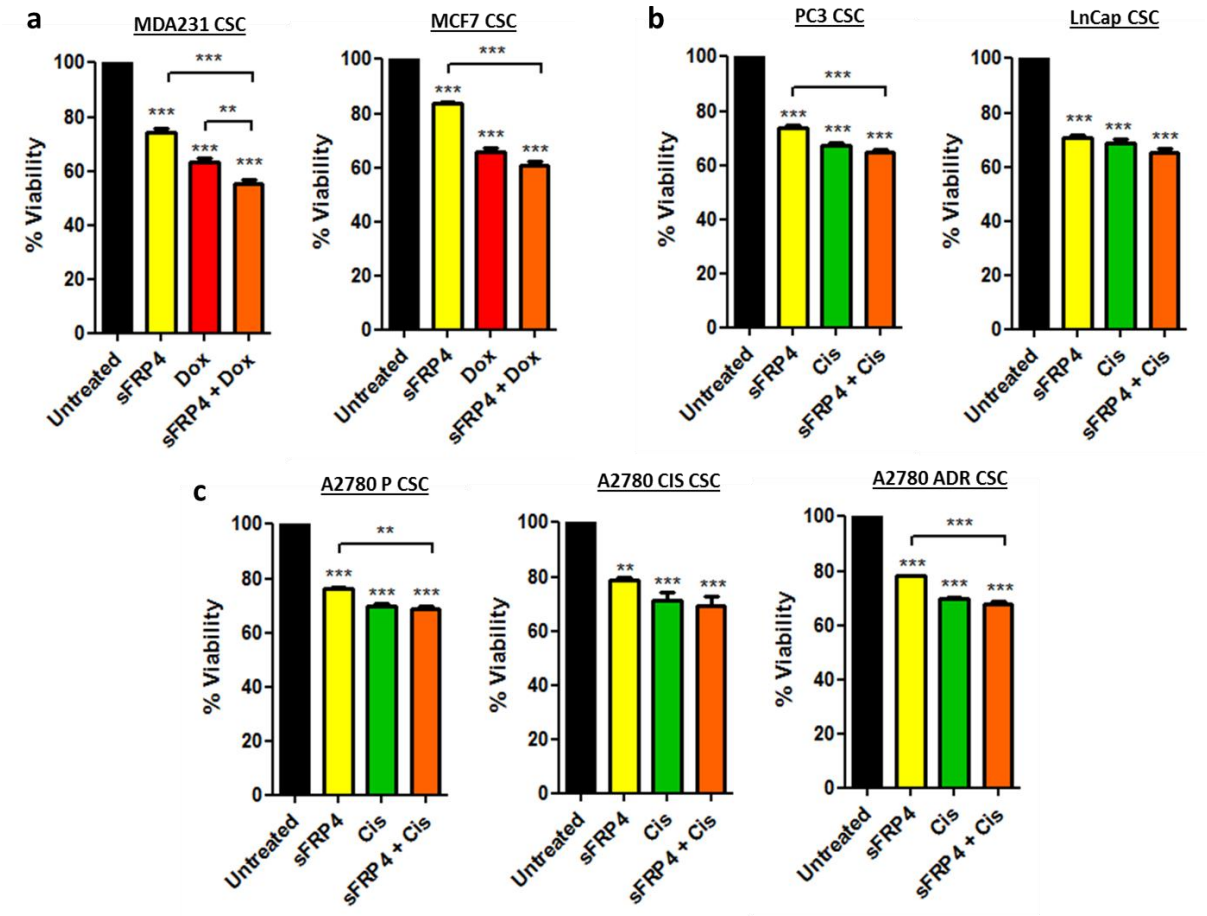


Figure 3: Effect of sFRP4 on CSC viability: Viability assay was performed by MTT after treatment of CSCs derived from A) Breast tumor cell line-derived CSCs (MDA231/ MCF7). B) Prostate tumor cell line-derived CSCs (PC3/ LnCap). C) Ovary tumor cell line-derived CSCs (A2780 P/ CIS/ ADR) with sFRP4 alone or in combination with Doxorubicin/Cisplatin for 24 hr. Statistical analysis was performed using ANOVA for analysis variance with Bonferroni test for comparison showing significance as ***P<0.001; **P<0.01; *P<0.05. Data are mean ± standard error of the mean from 3 independent experiments.

2.2.4 SFRP4 and Doxorubicin/Cisplatin treatment downregulates the expression of CSC stemness genes

The stemness related genes *SOX2*, *Klf4*, *Nanog*, and *Oct4* are expressed in CSCs and are associated with tumor progression. Semi-quantitative PCR analysis showed the untreated CSCs expressing all the genes, but the treatment with sFRP4 alone or in combination with Doxorubicin/Cisplatin downregulated the expression of *SOX2*, *Klf4*, *Nanog*, and *Oct4* in all the cell line-derived CSCs. The combinatorial treatment showed maximum reduction of gene expression, indicating that sFRP4 in combination with chemotherapeutic drugs has the capacity to reverse the stem cell-like properties of CSCs (Figure 4).

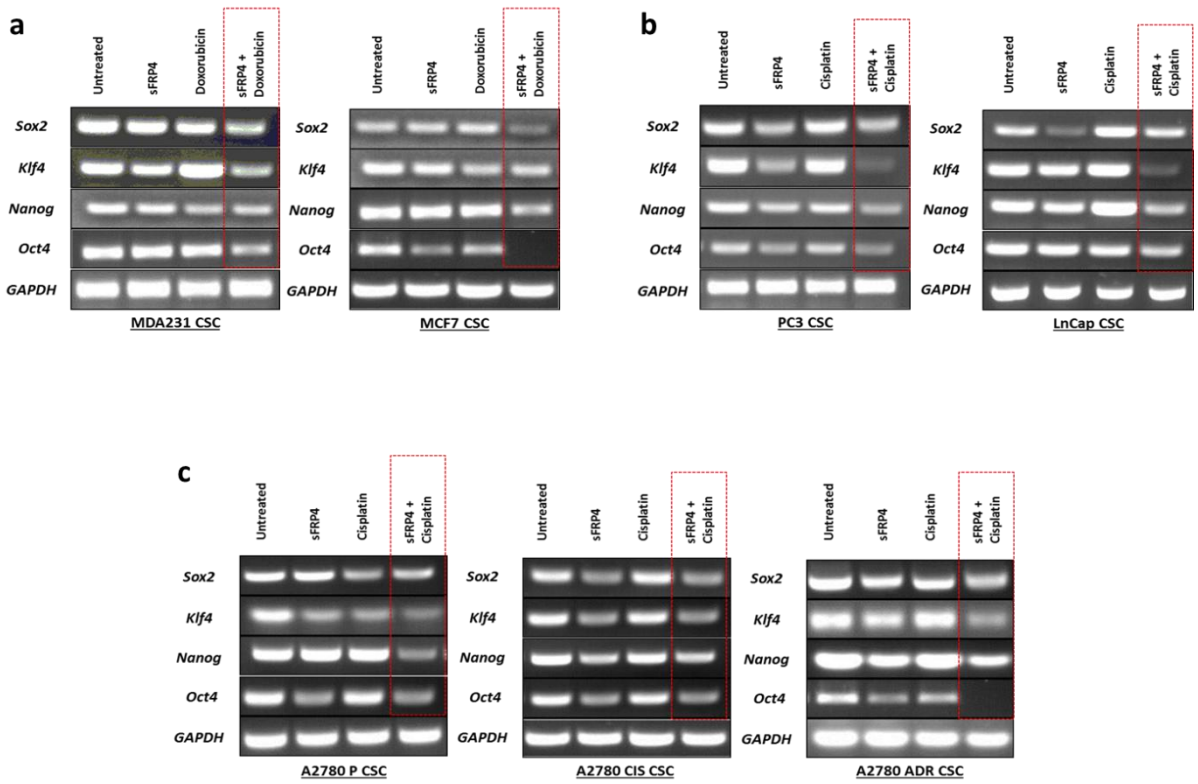


Figure 4: Effect of sFRP4 on CSC stemness gene expression: sFRP4 in combination with chemotherapeutic drugs (Doxorubicin/Cisplatin) reduced the expression of stemness-related genes, indicating loss of stem-like expression and differentiation capacity. Semi-quantitative PCR images are representative of 3 experiments.

2.2.5 SFRP4 mediates early apoptotic events in CSCs

The disruption of mitochondrial membrane potential was investigated by using JC-1 dye. Results from the JC-1 assay demonstrated a significant increase ($p<0.01$) in mitochondrial depolarization after treatment with sFRP4, Doxorubicin/Cisplatin alone, and in combinatorial treatments compared to untreated control. In all cell line-derived CSCs, maximum depolarization was observed in combinatorial treatments, indicating early stage death and apoptotic response through sFRP4 (Figure 5a). To further confirm the apoptotic role of sFRP4 in CSCs, we studied caspase 3 activity in CSCs derived from all cell lines, which indicated increased caspase 3 activity ($p<0.001$) in the sFRP4 alone and combinatorial treatments in comparison to untreated cells (Figure 5b).

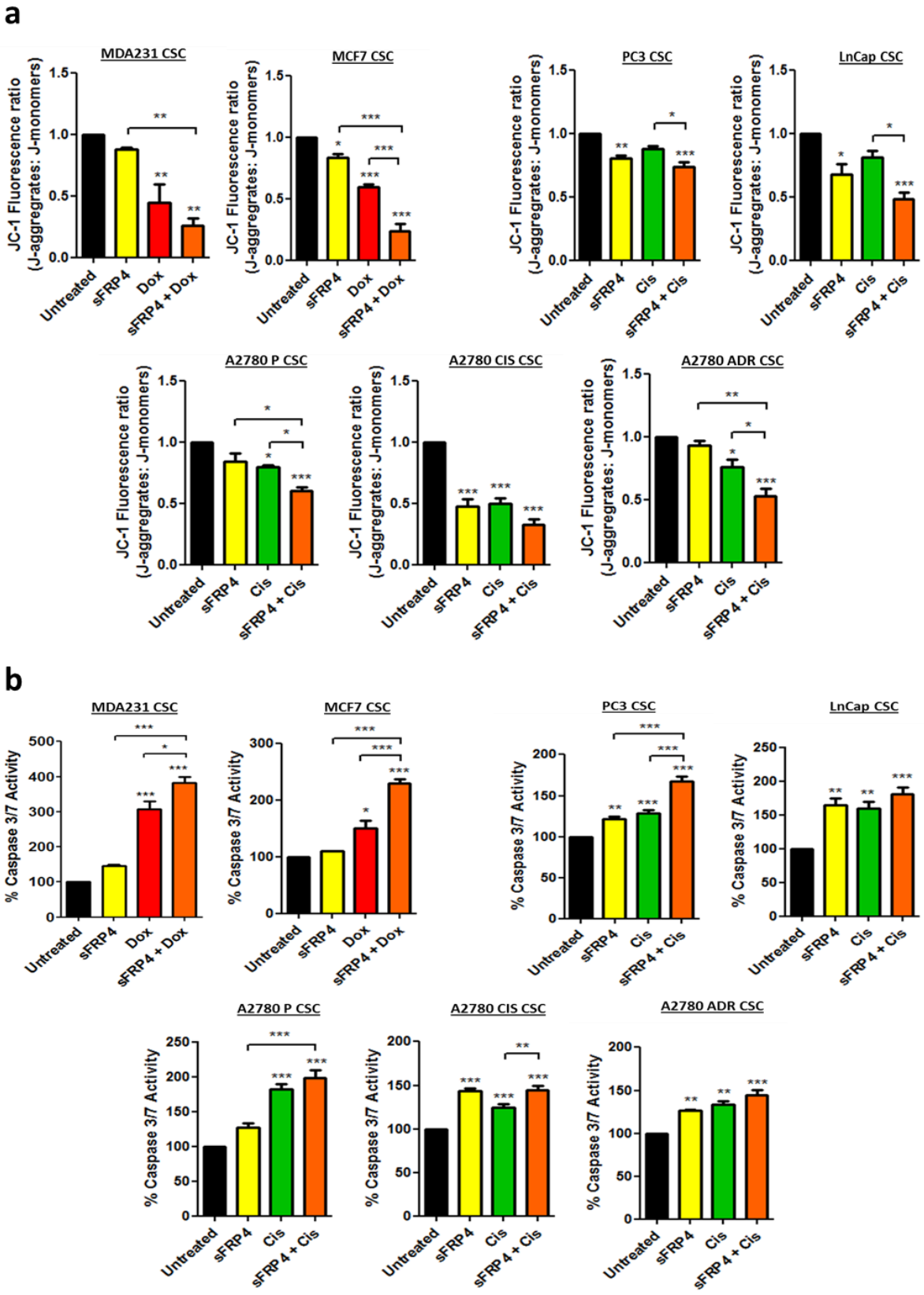
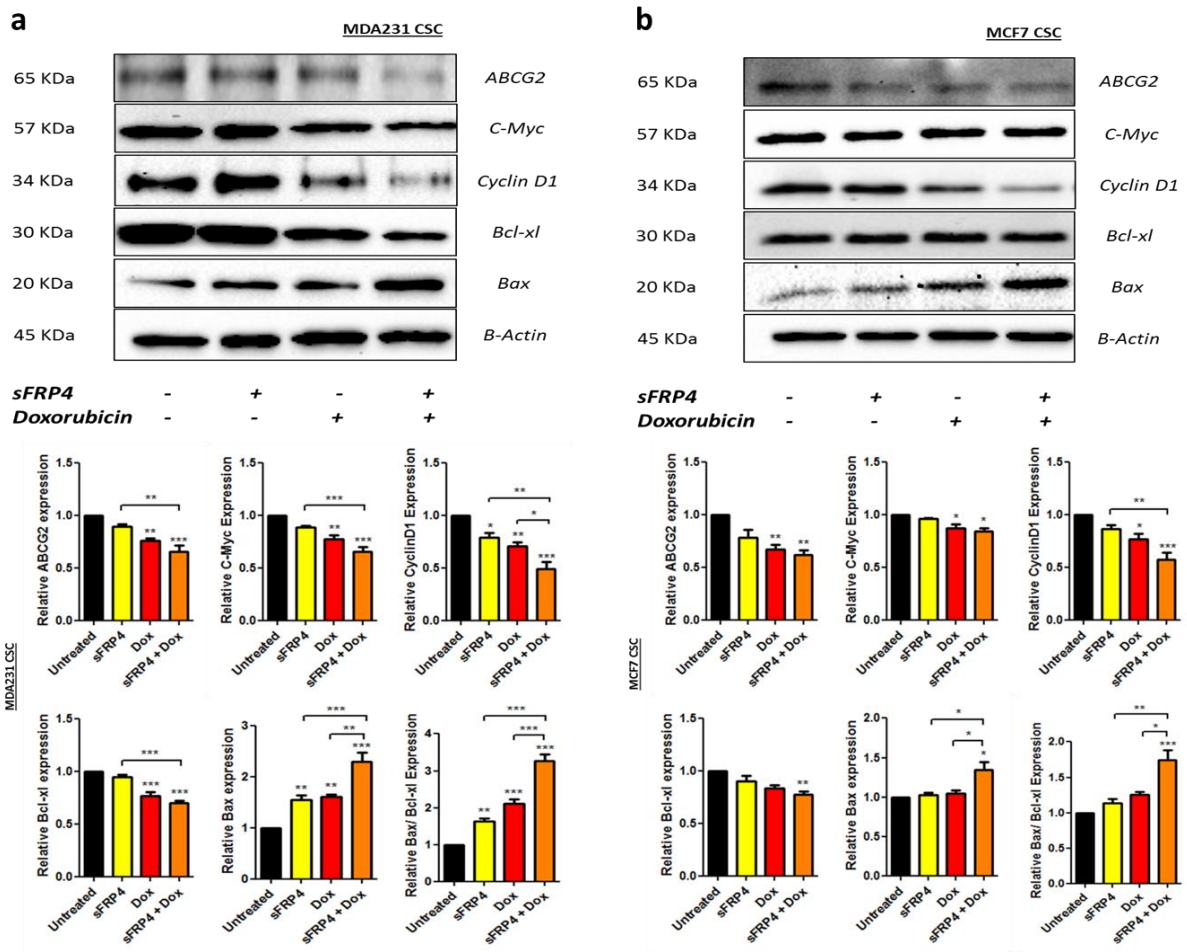
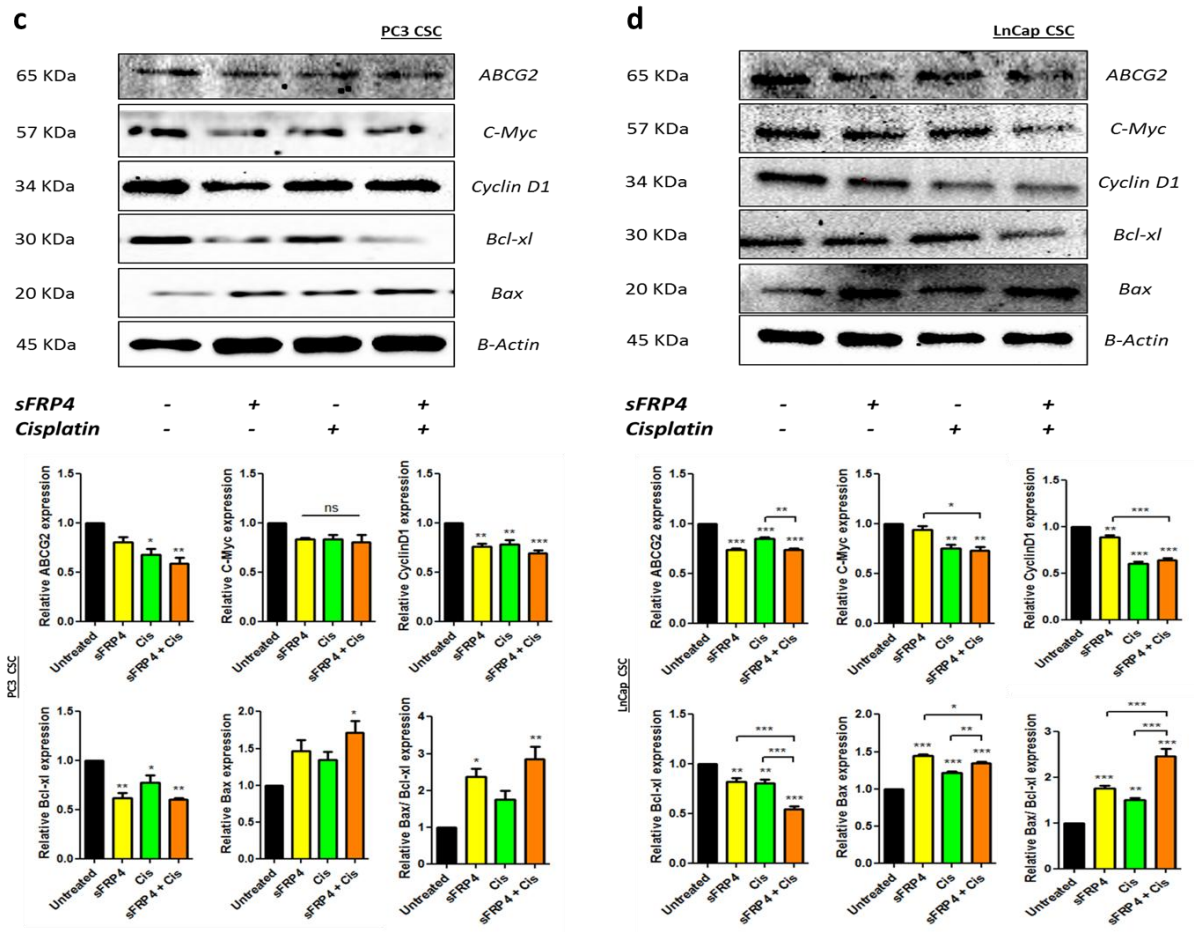

a
MDA231 CSC
MCF7 CSC
PC3 CSC
LnCap CSC
A2780 P CSC
A2780 CIS CSC
A2780 ADR CSC
JC-1 Fluorescence ratio (J-aggregrates: J-monomers)
Untreated
sFRP4
Dox
sFRP4 + Dox
Cis
sFRP4 + Cis
b
% Caspase 3/7 Activity

Figure 5: sFRP4 initiates early apoptotic events in CSCs. A) Detection of JC-1: The JC-1 assay demonstrated a high mitochondrial depolarization in sFRP4, Doxorubicin/Cisplatin, and combination-treated CSCs, indicating early stage cell death and apoptotic response to the various drug treatments. The combinatorial treatment on CSCs showed maximum depolarization. B) Detection of caspase-3 activity: Increasing amount of caspase-3 substrate indicates initiation of apoptosis. The caspase-3 activity (an indicator of late apoptosis) of CSCs was significantly upregulated in combinatorial treatment compared to untreated cells. Statistical analysis was performed using ANOVA for analysis variance with Bonferroni test for comparison showing significance as ***P<0.001; **P<0.01; *P<0.05. Data are mean ± standard error of the mean from 4 independent experiments.

2.2.6 SFRP4 regulates protein expression in CSCs

Following sFRP4 treatment, we investigated the post-translational modifications in CSCs for ABC transporters (ABCG2), oncogenes (*c-Myc*), anchorage independent cell survival (Cyclin D1), anti-apoptotic (Bcl-xl), and pro-apoptotic (20) proteins. Results demonstrated the existing chemo-resistance of CSCs (Figure 6). ABCG2 was highly expressed in the untreated groups but decreased in the presence of sFRP4, Doxorubicin/Cisplatin, and combinatorial treatments, with the latter inducing the lowest expression levels. *c-Myc* had similar expression levels in all CSCs, except ovarian A2780-Cis CSCs (Figure 6g). The levels of the proto-oncogene cyclin D1 decreased in all the combinatorial treatments of CSCs compared to untreated CSCs, except in prostate PC3 CSCs (Figure 6c). Overexpression of the anti-apoptotic protein Bcl-xl in untreated CSCs confers chemo-resistance; however, the combinatorial treatment produced a significant decrease in protein expression levels, indicating sFRP4's pro-apoptotic capacity. Expression of pro-apoptotic protein Bax was lower in untreated CSCs but increased significantly with combinatorial treatment. The increased Bax/Bcl-xl expression level ratio confirms the pro-apoptotic role of sFRP4.


a
MDA231 CSC
65 KDa
57 KDa
34 KDa
30 KDa
20 KDa
45 KDa
ABCG2
C-Myc
Cyclin D1
Bcl-xl
Bax
B-Actin
sFRP4 - + - +
Doxorubicin - - + +
b
MCF7 CSC
65 KDa
57 KDa
34 KDa
30 KDa
20 KDa
45 KDa
ABCG2
C-Myc
Cyclin D1
Bcl-xl
Bax
B-Actin
sFRP4 - + - +
Doxorubicin - - + +
MDA231 CSC
MCF7 CSC
Relative ABCG2 expression
Relative C-Myc Expression
Relative CyclinD1 Expression
Relative Bcl-xl expression
Relative Bax expression
Relative Bax/ Bcl-xl Expression
Untreated
sFRP4
Dox
sFRP4 + Dox


c
PC3 CSC
65 KDa
57 KDa
34 KDa
30 KDa
20 KDa
45 KDa
ABCG2
C-Myc
Cyclin D1
Bcl-xl
Bax
B-Actin
sFRP4 - + - +
Cisplatin - - + +
d
LnCap CSC
65 KDa
57 KDa
34 KDa
30 KDa
20 KDa
45 KDa
ABCG2
C-Myc
Cyclin D1
Bcl-xl
Bax
B-Actin
sFRP4 - + - +
Cisplatin - - + +
PC3 CSC
LnCap CSC
Relative ABCG2 expression
Relative C-Myc expression
Relative CyclinD1 expression
Relative Bcl-xl expression
Relative Bax expression
Relative Bax/ Bcl-xl expression
ns
Untreated
sFRP4
Cis
sFRP 4 + Cis

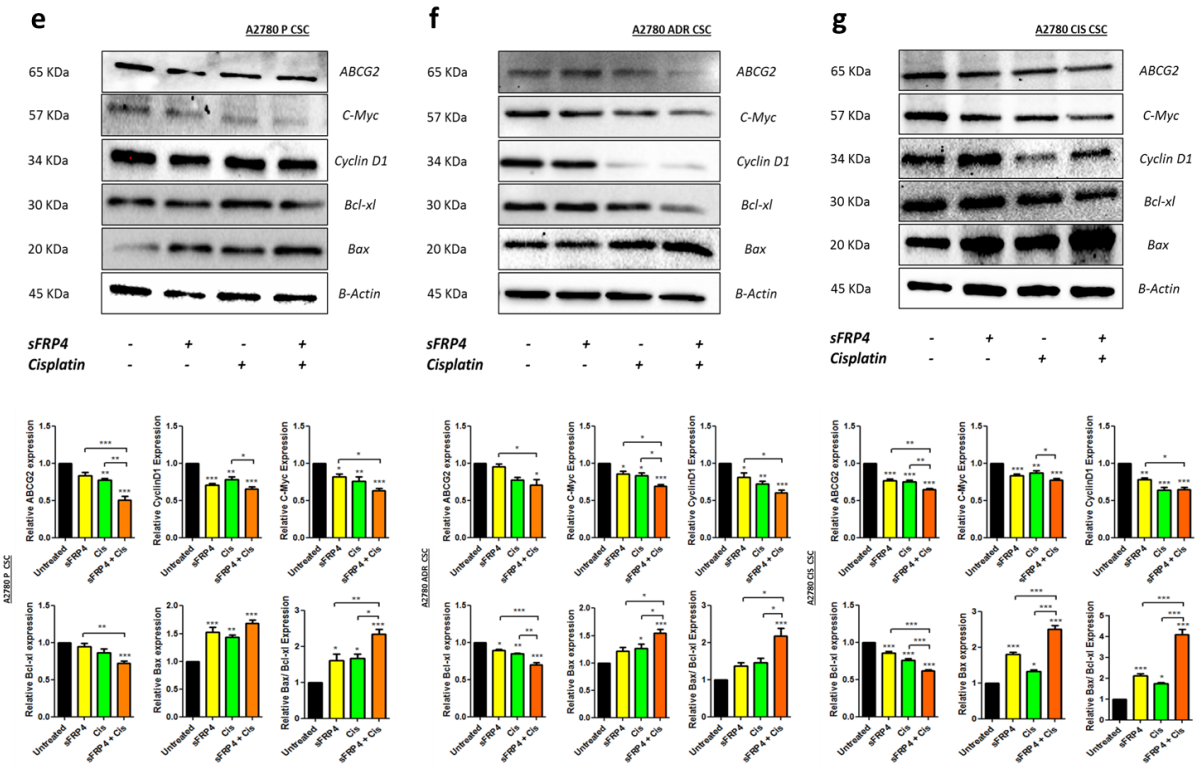


Figure 6: Effects of sFRP4 on protein expression levels: The combinatorial treatment of sFRP4 and chemotherapeutic drugs significantly upregulated the apoptotic protein (BAX) and downregulated cell survival (Cyclin D1) and oncogenes (C-Myc). A/B) Breast tumor cell line-derived CSCs (MDA231/ MCF7). C/D) Prostate tumor cell line-derived CSCs (PC3/ LnCap). E/F/G) Ovary tumor cell line-derived CSCs (A2780 P/ CIS/ ADR). In combinatorial treatment, an elevated BAX/ Bcl-xl ratio corresponded to elevated cell apoptosis. Statistical analysis was performed using ANOVA for analysis variance with Bonferroni test for comparison showing significance as ***P<0.001; **P<0.01; *P<0.05. Blots and relative protein expressions are mean ± standard error of the mean from 3 independent experiments.

2.3 Discussion

The role of CSCs in solid tumors is well established (21-23). CSCs are in a quiescent state and remain in the resting phase of the cell cycle (G0 phase), expressing high levels of drug efflux transport systems (24, 25). Due to the CSCs' dormant state, chemotherapeutic drugs are unable to target CSCs, whereas they kill only proliferative tumor cells that are in M phase (26, 27). Therefore, the knowledge of chemo-resistance associated with CSCs is of great importance for our understanding of reproductive tumors (e.g. breast, prostate, and ovary tumors), which are tumors with poor prognosis. In order to gain further insights into the chemo-sensitization of CSCs, we investigated the role of sFRP4 when used in combination with the chemotherapeutic drugs Doxorubicin and Cisplatin on CSCs derived from various tumor cell lines. Our study demonstrates sFRP4 alone or in combinatorial treatment with chemotherapeutic drugs elicits anti-proliferative effects, spheroid disruption, decrease in cell survival, and initiation of apoptosis within the CSCs, therefore indicating chemo-sensitization.

The CSCs were identified from breast (MDA231 and MCF7), prostate (PC3 and LnCap), and ovary (A2780 P, A2780 ADR, and A2780 CIS) tumor cell lines based on their ability to form spheroids in serum free conditions, elevated expression of CSC surface markers, high expression of ABC drug transporters (ABCG2), cell survival protein (Cyclin D1), oncogenes (c-Myc), and the ability to escape cell death/apoptosis (Bcl-xl). The resistance in CSCs is often correlated to the CSC surface marker profiles; $CD44^{+High}$ and $CD133^{+High}$ cells are highly radio-resistant in colon cancer and they have a higher DNA repair capacity and ability to escape apoptosis compared to $CD44^{Low}$ and $CD133^{Low}$ CSCs (28). Targeting $CD44^{+}$ cells demonstrated higher anti-proliferative activity *in-vitro* compared to the anti-tumor drug SN38 when used against colon, gastric, breast, oesophageal, lung, and ovarian cancer cells (29, 30). A reduction in the expression of CSC markers was observed in our study, where we demonstrated that sFRP4 in combination with chemotherapeutic drugs was able to decrease the $CD44^{+}$ 24^{-} population for breast-derived CSCs, and the $CD44^{+}$ $CD133^{+}$ population for prostate and ovary-derived CSCs. In ovarian A2780 P derived CSCs, the Cisplatin treatment aberrantly increased the $CD133^{+}$ $CD44^{+}$ population, and decreased the $CD44^{+}$ and $CD133^{+}$ alone population; this aberration could imply that phenotypic switching of CSCs has occurred, a process that is still not well understood (31).

Clinically, CSCs reside in anatomically distinct regions within the tumor microenvironment (known as niches), which preserve the CSCs' phenotypic plasticity, facilitates metastatic potential, and supports high expression of drug efflux transporters, making them highly chemo-resistant (32). Although CSC isolation and characterization has been studied extensively, *in-vitro* CSCs niches are characterized by spheroid forming capacity in serum free conditions (33). We showed that targeting the Wnt signalling pathway by using sFRP4 has the capacity to disrupt the niches when sFRP4 was used in combination with chemotherapeutic drugs. Spheroid disruption by sFRP4 decreases the CSCs' plasticity and cell-cell adhesion, initiating the CSCs' differentiation towards tumor cells and reducing their self-renewal capacity. This opens the gateway for chemotherapeutic drugs to target the cells at high potency. We confirmed spheroid disruption using immunofluorescence, and we observed the spheroid disruption was associated with reduced CSCs' marker profile. We also observed a marked reduction of the proliferation marker Ki-67 and drug transporter ABCG2 in sFRP4 combinatorial treatment. The prostate cell line LnCap-derived CSCs showed an absence of $CD44^{+}$ expression, which is in agreement with previous work (34).

In previous studies, sFRP4 has shown an anti-proliferative capacity in CSCs derived from glioblastoma multiforme, and head and neck tumor (19, 35). In this study we have demonstrated that sFRP4 in combination with chemotherapeutic drugs decreased the viability of CSCs compared to drug treatment alone, indicating sFRP4s' role in the increased chemo-response of CSCs.

The Wnt signalling pathway has been reported to regulate stemness in CSCs derived from colon cancer (36) and breast cancer (37), although Wnt activation is higher in breast CSCs compared to normal stem-like cells (38). Genes controlling the stemness of CSCs have distinct functions and are important for CSC development and self-renewal, and are responsible for replicative quiescence (39). We hypothesized that cancer stemness inhibition can effectively suppress metastatic potential and tumor recurrence, although gene profiling of cancer stemness is more similar to embryonic stem cells than adult stem cells (40). In this study, we show that sFRP4 reduced the expression of various stemness genes including *Sox2, Klf4, Nanog*, and *Oct4* when treated in combination with chemotherapeutic drugs. These genes encode key stemness transcription factors that are

important for maintenance of pluripotency (41, 42). These data demonstrate the role of sFRP4 in inhibiting CSCs by modulating stemness gene expression.

Chemo-resistance of CSCs is due to alterations in expression of anti-apoptotic (Bcl-2 family) and pro-apoptotic genes (20, 43). Apoptosis can be triggered by two pathways: a) the extrinsic pathway where caspase-8 activation is initiated by the ligand of death receptors on the cell surface; and b) the intrinsic pathway where mitochondria release *cytochrome c* (44). The release of *cytochrome c* is a crucial step that activates caspase-9 by assembling the apoptosome, further activating the downstream executioners caspase 3/7 (45). In previous studies, the relationship between sFRP4 and apoptosis has been identified, demonstrating sFRP4 as a pro-apoptotic agent (18, 46). This was further confirmed by assessing the integrity of the mitochondrial membrane when the CSCs were treated with sFRP4. We observed the mitochondrial depolarization by application of the JC-1 assay, as low $\Delta\psi_M$ (mitochondrial membrane potential) due to depolarization is indicative of apoptosis, indicating sFRP4 chemo-sensitzation involves the initiation of apoptotic pathways. We further demonstrated the pro-apoptotic role of sFRP4 with an elevation in caspase 3/7 expression in CSCs treated with sFRP4 alone or in combination with chemotherapeutic drugs, indicating the later onset of apoptosis.

The overexpression of Bcl-2 is associated with chemo-resistance. The 3D protein structure of Bcl-xl revealed the structural similarities within the Bcl-2 family, possessing 4 BH domains and promoting cell survival by inactivation of Bcl-2 counterparts and preserving outer mitochondrial membrane integrity (47). One of the early studies to show the chemo-sensitizing effects targeting Bcl-2 involved treating patients with Bcl-2 antisense (oblimersen sodium) in combination with chemotherapeutic drugs in chronic lymphocytic leukemia, leading to improved survival (48, 49). We hypothesized that sFPR4 had the potential to bind the hydrophobic groove of anti-apoptotic Bcl-2, which would oligomerize Bax and can subsequently lead to mitochondrial membrane potential to depolarize releasing *cytochrome c*. We demonstrated a gradual increase in Bax expression and decreased Bcl-xl expression with sFRP4 and chemotherapeutic drug treatment alone; however, the combinatorial treatment elevated Bax and inversed the effect on Bcl-xl. The Bax/Bcl-xl ratio is an indicator for apoptosis, and an increased ratio depicts the activation of caspase 3 (50). The Bax/Bcl-xl ratio was consistently high in all CSCs treated with combinatorial treatment. The elevated

expression of apoptotic genes within all the CSCs indicates sFRP4's role as a pro-apoptotic agent.

We also observed a decrease in Cyclin D1 expression, which is an oncogene driving cell cycle progression. Cyclin D1 interacts with proteins involved with DNA repair, RNA metabolism, and cell structure; deregulation of Cyclin D1 will affect cellular processes and eventually lead to an inefficient DNA damage repair system (51). In the Wnt signalling pathway, GSK-3β regulates Cyclin D1 degradation, and the gene expression is activated by Wnt signalling (52, 53). SFRP4 is a Wnt antagonist, which binds to the frizzled receptors and activates the GSK-3β destruction complex, initiating the degradation of Cyclin D1. In contrast, *c-Myc* expression showed no decrease in A2780 CIS, PC3, and MCF7-derived CSCs. c-Myc is an oncoprotein that is an important regulator in stem cell biology (54) and correlates to tumor metastasis (40). CSCs exhibit a high expression of c-Myc, and downregulation of c-Myc leads to apoptosis under various circumstances (55-58). We hypothesize that a different dose of sFRP4 and chemotherapeutic drugs would enable a reduction in *c-Myc* expression.

In summary, sFRP4 chemo-sensitizes CSCs derived from breast, prostate, and ovary tumor cell lines by reducing their pro-oncogenic profile, stemness capacity, cell survival protein and oncogene expression, making them more responsive to chemotherapeutic drugs. Chemo-sensitization by sFRP4 *in vivo* may decrease the required chemotherapeutic load required to reduce the tumor mass. SFRP4 prevents a sustained Wnt inhibition in order to provide a therapeutic window for chemotherapy while sparing normal Wnt-dependent tissues. Further *in vivo* studies may confirm the role of sFRP4 in the chemo-sensitization of CSCs to prevent tumor relapse and lead to tumor resolution.

2.4 Materials and Methods

2.4.1 Monolayer cell culture

Cell culture plates for adherent cells were purchased from Nunc™ (ThermoFisher Scientific). The human breast cells line MDA-MB 231 (ER-) and MCF-7 (ER+), human ovary cell lines A2780-P, A2780-ADR, and A2780-Cis, and human prostate cell lines PC-3 (AR-/PSA-) and LnCap (AR+) were purchased from American Type Culture Collection (ATCC, USA). The cells were cultured in RPMI-1640 medium (Gibco #11875-093) supplemented with 10% foetal bovine serum (Bovogen #SFBS) and 100 U/ml PenStrep (Life Technologies #15070063). All cells were maintained at 37°C in a humid incubator with 5% CO_2.

2.4.2 Cancer stem cell isolation

For CSC isolation, culture plates with an ultra-low-attachment surface were purchased from Corning Life Sciences. CSCs were cultured in serum-free medium (SFM) containing basal medium RPMI-1640 + DMEM-HG (HyClone, USA #SH30081.02) supplemented with the growth factors bFGF (20 ng/ml) (ProSpec Bio #cyt-085), EGF (20 ng/ml) (ProSpec Bio #cyt-217), and 1× B27 (Gibco #17504044), and 100 U/ml PenStrep (Life Technologies #15070063). CSC-enriched populations of cells were obtained by plating a single cell suspension of breast, ovary, and prostate tumor cells at 10000 cells/cm² in SFM on Low-adherent six-well plates (Corning #3471). CSCs were isolated in SFM; the spheroids are formed at 3rd Day of plating tumour cells. The sFRP4 (250pg/ml) and Chemotherapeutic drugs alone or in combination were added on 3rd Day for 24 hours. Post 24 hour treatment; the spheroids were dissociated and maintained in CSC culture medium in low-attachment-surface plates for functional studies.

2.4.3 Chemo-Sensitization/Drug treatment

The drugs used in this study were purified sFRP4 (R&D Systems #1827-SF-025), Doxorubicin (Sigma #D1515), and Cisplatin (Sigma #P4394). CSC sensitization with sFRP4 was performed by adding sFRP4 to the cell culture at 250 pg/ml (59) for 24 hr at 37°C in 5% CO_2 incubator. Doxorubicin was tested at an IC50 value of 1 - 10 µM. Cisplatin was tested at its IC50 value of 10 – 50 µM. The drug treatment for the downstream analysis was optimized at 250 pg/ml of sFRP4 alone or in combination with 5 µM of doxorubicin (breast tumor cells only) and 30

µM of cisplatin (ovary and prostate tumor cells only) for 24 hr. An MTT assay was used for the analysis of cellular viability.

2.4.4 Cell surface markers

To assist in determining their identity, cell surface markers (Table 2) were examined in both monolayers and CSCs by flow cytometry (BD FACSCANTO II) using CellQuest data acquisition and analysis software. APC-CD44 (1:100) (BioLegend #338805), PE Cy7-CD24 (1:10) (BioLegend #311119), and PE-CD133 (1:100) (BioLegend #372803). Cells incubated with conjugated irrelevant IgGs were used as negative controls.

Table 2: Tumor specific cancer stem cell markers

Cancer	CSC Markers	Reference
Breast	$CD24^{-/low}$/ $CD44^{+}$	[3,59]
Ovary	$CD133^{+}$/ $ALDH1^{+}$	[60]
Prostate	$CD133^{+}$/ $CD44^{+}$/ $ABCG2^{+}$	[61]

2.4.5 Immunofluorescence staining

The spheroids were plated on Poly-D-lysine pre-coated 96 well plates (Sigma #6407), incubated at 37°C for 3 hr, and then fixed in 4% paraformaldehyde (Sigma #P6148) overnight at 4°C. The cells were washed three times with PBS, incubated for 1 hr in 1% BSA Blocking buffer, and incubated with primary antibodies CD44 (Cell Signaling #3570); CD24 (ThermoFisher #MA5-11828); CD133/1 (Miltenyi #130-090-422); ABCG2 (Cell Signaling #42078); Ki-67 (Millipore #AB9260) overnight at 4°C. After three 10 mins washes with PBS, the cells were incubated with the appropriate secondary antibody (see Supplementary Table 1) for 1 hr at room temperature. After three 10 mins PBS washes, cells were incubated with Hoechst 33342 (Sigma #14533) at 1:20000 dilution at room temperature for 15 mins. The cells were then washed with PBS three times for 5 mins each and observed using an Ultraview Vox spinning disk confocal microscope (Perkin Elmer).

2.4.6 MTT viability assay

Cell viability kit - an MTT kit (Sigma #M5655) was used to measure cell metabolic viability.

Monolayers - 5000 cells/cm² were plated in a flat-bottomed 96-well plate for 2 days with culture medium. After that, sFRP4 alone or in combination with the tumor-specific drug was added and the cells were incubated for 24 hr. After drug treatment, the MTT assay was performed as per the manufacturer's instructions.

CSCs - 10000 cells/cm² of monolayer cells were plated in a low-adherent flat-bottomed 96-well plate (Corning #3474) for 3 days in non-adherent SFM conditions. After that, sFRP4 alone or in combination with the tumor-specific drug treatment was added and the cells left for 24 hr, following which the MTT assay was performed. Plates were read at 595 nm using an EnSpire Multilabel Plate Reader (Perkin-Elmer).

2.4.7 Reverse transcription-polymerase chain reaction

Total RNA was isolated from cells using TRIzol reagent (Life Technologies #15596026) followed by chloroform extraction, isopropanol precipitation, and a 75% (v/v) ethanol wash. RNA samples (1 µg) were reverse-transcribed to cDNA using a High Capacity cDNA kit (Applied Biosystems #4368814). cDNA in 1 µl of the reaction mixture was amplified with PCR Master Mix (Life Technologies #K0171) and 10 µM each of the sense and antisense primers. The thermal cycle profile was as follows: denaturation at 95 °C for 30 s, annealing at 55-61°C for 30 s depending on the primers used, and extension at 72 °C for 90 s. Each PCR reaction was carried out for 35 cycles, and the PCR products were size fractionated on 1% agarose gel/ GelGreen (1:10000) (Fisher Biotec #41005) and visualized under UV Trans illumination (FB Biotech). The primer sequences are described in Supplementary Table 2.

2.4.8 Western blotting

Cells were washed twice with PBS and then lysed in RIPA lysis buffer (Sigma #R0278) (150 mM NaCl, 1.0% IGEPAL® CA-630, 0.5% sodium deoxycholate, 0.1% SDS, 50 mM Tris, pH 8.0, Proteinase Inhibitor 1x). Post sonication, cell lysates were centrifuged at 14000g for 10 min at 4 °C, and the supernatants were used for Western blotting. The lysates were resolved by sodium dodecyl sulfate-polyacrylamide gel electrophoresis, transferred onto nitrocellulose membranes, and then stained with 0.1% Ponceau S solution (Sigma #P3504) to ensure equal loading of the samples. After being blocked with 5% non-fat milk for 60 mins, the

membranes were incubated with primary antibodies ABCG2 (Cell Signaling #42078); c-Myc (Cell Signaling #5605); Cyclin D1 (Abcam #ab134175); Bcl-xL (Cell Signaling #2764); Bax (Cell Signaling #5023); β-Actin (Cell Signaling #4970) overnight, and the bound antibodies were visualized with horseradish peroxidase-conjugated secondary antibodies using the ECL Western Blotting Substrate (Amersham, GE #RPN2106) on a Chemi-Doc (Bio-Rad) imaging analyzer. The primary antibodies concentrations are described in Supplementary Table 3.

2.4.9 Apoptotic assays

JC-1 Assay: $\Delta\psi_M$ is an important parameter of mitochondrial membrane and has been used as an indicator of cell health. JC-1 enters the mitochondria and changes its fluorescent properties based on aggregation of the probe, and forms complexes known as J-aggregates with intense red fluorescence. High $\Delta\psi_M$ predicts healthy cells and low $\Delta\psi_M$ exhibits mitochondrial membrane potential depolarization indicative of apoptosis. JC-1 activity was measured using JC-1 Mitochondrial Membrane Potential Assay Kit (Cayman #10009172). CSCs were enriched in 96 well low attachment plates, and treated with sFRP4 alone and in combination with chemotherapeutic drugs for 24 hr and compared with untreated CSCs. Post-treatment, 10µl of JC-1 staining solution is added to each well and incubated at 37°C for 30 mins. The plate was washed with assay buffer at 400g for 5 mins twice. This was followed by addition of 100µl of assay buffer to each well and read using an EnSpire Multilabel Plate Reader (Perkin-Elmer) for analysis. JC-1 aggregates were measured at excitation and emission wavelengths of 535nm and 595nm respectively. JC-1 monomers were measured at excitation and emission wavelengths of 485nm and 535nm. The ratio of fluorescent intensity of J-aggregates and J-monomers (Red: Green) was used as an indicator of cell health. The JC-1 assay kit is highly light sensitive and all procedures were conducted in dark conditions.

Caspase-3 Assay: Caspase-3 activity was measured using the EnzChek Caspase-3 Assay Kit II (Molecular Probes #E13184). Briefly, 50 µl of the supernatant was added to an individual well of a 96-well micro fluorescent plate and incubated for 10 min at room temperature. After incubation, 50 µl of the 2x working substrate (5 M Z-DEVD-R110) were added to each well and further incubated for 30 min at 37°C. Fluorescence was measured at 485 nm excitation and 538 nm emissions using an EnSpire Multilabel Plate Reader (Perkin-Elmer). Caspase-3 activity was expressed as arbitrary units of fluorescence.

2.5 Statistics

Statistical analysis was performed with GraphPad Prism V5.0 (GraphPad software, La Jolla, USA) using one-way ANOVA for analysis variance with Bonferroni test for comparison showing significance as ***P<0.001; **P<0.01; *P<0.05. Data are presented as mean ± standard error of the mean.

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Author contributions: AbhiD drafted the outline and generated the data. SK contributed the viability data. AbhiD wrote the manuscript. AbhiD and ArunD conceived the study, and ArunD, FA, and PN critically reviewed, revised, and approved the final manuscript.

Competing financial interests

The authors declare that they have no competing interests.

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Chapter 3

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3 Novel peptides derived from secreted frizzled-related protein 4 increase chemotherapeutic outcomes in breast, prostate, and ovarian cancer stem cells.

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Cancer stem cells, Wnt signalling pathway, secreted fizzled-related protein, Chemo-sensitisation, Chemo-resistance, Peptides, Apoptosis.

Abbreviations:

CSCs: Cancer stem cells; sFRP: Secreted frizzled-related protein; LSCs: Leukaemic stem cells; GSK3β: Glycogen synthase kinase 3 beta; MTT: 3-(4,5-Dimethylthiazol-2-yl)-2,5-Diphenyltetrazolium Bromide.

Abstract

Chemotherapy is the most common treatment for cancer, though most tumour cells can acquire chemo-resistance towards the treatment. We have previously demonstrated that secreted frizzled-related protein 4 (sFRP4, composed of 340 amino acids), a Wnt antagonist, confers chemo-sensitisation and chemotherapeutic efficacy within the tumour subset population known as cancer stem cells (CSCs). In this study, we have investigated the effects of sFRP4-derived peptides (SC301 and SC401, 15-40 amino acids in size) alone or in combination with tumour-specific drugs on CSCs derived from breast, prostate, and ovary tumour cell lines. Combinatorial treatment of peptides and tumour-specific drugs resulted in enhanced cytotoxicity of doxorubicin and cisplatin. The CSC chemo-sensitisation induced by SC301 and SC401 was dependent upon decreased expression of drug efflux proteins and proto-oncogenes, and increased activation of pro-apoptotic proteins. Our results suggest that the combinatorial treatment of SC301 and SC401 with tumour-specific drugs effectively chemo-sensitises the CSCs by decreasing CSC viability and negatively impacting the molecular signals responsible for CSC survival and self-renewal capacity. The peptides demonstrated higher chemo-sensitisation efficacy compared to the whole sFRP4 protein. As such, the sFRP4-derived peptides provide a promising new approach for site-specific treatments, improved *in-vivo* chemo-response, and patient prognosis.

3.1 Introduction

Chemotherapeutic drugs play a significant role in cancer treatment and decreasing tumour progression (1). However, Chemo-resistance, whether inherent or acquired, greatly threatens clinical outcomes and patient survival. Progress in finding a potent agent to enhance cancer chemo-sensitivity has been slow, perhaps related to poor outcomes associated with past clinical trials (2).

Numerous molecular markers indicate various levels of chemosensitivity, though they are not ideal for developing tumour sensitising drugs (3). Chemo-resistance has often been associated with the presence of a tumour sub-set population with self-renewal capacity, known as cancer stem cells (CSCs). CSCs are a heterogeneous, distinct population within the tumour mass and exhibit long-term clonal repopulation (4, 5). These CSCs are equipped with apoptotic insensitivity, high drug efflux transporter expression, and DNA repair and quiescence, which make them highly chemo-resistant (6, 7). Considering these important features of CSCs, inhibiting CSCs is perhaps a higher priority than killing rapidly proliferating cells. A combinatorial treatment of tumour-specific drug with a CSC sensitiser may provide an efficient method of eliminating tumours in patients. In addition to their self-renewal and differentiation capacity, accumulating evidence indicates that CSCs from all malignancies are relatively resistant to chemotherapy and radiotherapy (8). Breast cancer patients who exhibit complete resistance to chemotherapy were found to have a higher baseline percentage of CSCs (9). Meanwhile, $CD44^{+}$/ $CD24^{-}$/low cells were 9.5 fold higher in breast cancer samples post chemotherapeutic treatment (10).

Activation of aberrant signalling pathways mediates the CSC chemo-resistance. Recently we demonstrated that Wnt/ β-catenin signalling is correlated to chemo-sensitivity (11-13). The Wnt signalling pathway plays a crucial role in mammary development and tumorigenesis (14, 15). In adult stem cells, intracellular β-catenin remains at normalized levels through proteasome degradation. However, β-catenin in CSCs could escape this degradation, leading to continuous nuclear transportation and downstream gene activation, which supports CSC survival and differentiation (16, 17). Wnt silencing has re-sensitised leukaemic stem cells (LSCs) to GSK3β inhibitors (18), and β-catenin deletion synergizes with imatinib to target resistant LSCs in chronic myeloid leukaemia (CML), and further β-catenin deletion suppresses CML recurrence even after imatinib withdrawal (19). Clinically, poor breast

cancer prognosis is associated with β-catenin accumulation in triple negative, basal-like type cancers (20). Targeting aberrant Wnt signalling in CSCs has potential to successfully chemo-sensitise the CSCs and reduce the clinical requirement and load of tumour-specific drugs.

Molecules for enhancing chemo-sensitivity should be target specific, potent, and relatively non-toxic to other cells in the body. We recently demonstrated that a combinatorial treatment using the Wnt signalling antagonist secreted frizzled-related protein 4 (sFRP4) with tumour-specific drugs chemo-sensitized CSCs derived from breast, ovary, and prostate tumour cell lines, and reduced the CSC population and inhibited aberrant Wnt signalling (11). Thus, therapeutic targeting of the Wnt signalling pathway is an effective means to eliminate CSCs (4).

In this study, we show for the first time, that small peptides (SC301 and SC401) derived from sFRP4 specifically target the formation and expansion of CSCs. We further provide evidence that these peptides chemo-sensitise CSCs to the clinically used anti-cancer drugs doxorubicin and cisplatin, inducing cytotoxicity via Wnt/β-catenin signalling regulation. Our *in vitro* study demonstrated that the peptides (15-20 amino acids) chemo-sensitise CSCs derived from breast, ovary, and prostate tumour cell lines via targeting the ATP-binding cassette subfamily G2 (ABCG2) protein, proto-oncogenes, anchorage-independent proteins, and anti-apoptotic protein. SC301 and SC401 in combination with chemotherapeutic drugs also demonstrated a pro-apoptotic effect on the CSCs, and also identified BAX and Bcl-xl as direct molecular targets of SC301 and SC401; thereby shedding light on the significance of target-specific treatment to abrogate cancer drug resistance and Wnt signalling in CSCs.

3.2 Materials and Methods:

3.2.1 Monolayer cell culture

Cell culture plates for adherent cells were purchased from Nunc™ (ThermoFisher Scientific). The human breast cancer cell lines MDA-MB 231 (ER^-) and MCF-7 (ER^+), human ovary cancer cell lines A2780-P, A2780-ADR, and A2780-Cis, and human prostate cancer cell lines PC-3 (AR^-/PSA^-) and LnCap (AR^+) were purchased from American Type Culture Collection (ATCC, USA). The cells were cultured in RPMI-1640 medium (Gibco) supplemented with 10% foetal bovine serum (Bovogen) and 100 U/ml PenStrep (Life Technologies). All cells were maintained at 37°C in a humid incubator with 5% CO_2.

3.2.2 Cancer stem cell isolation

For CSC isolation, culture plates with an ultra-low-attachment surface were purchased from Corning Life Sciences. CSCs were cultured in serum-free medium (SFM) containing basal medium RPMI-1640 + DMEM-HG (Hyclone, USA) supplemented with the growth factors bFGF (20 ng/ml), EGF (20 ng/ml) (ProSpec Bio), and 1× B27 (GIBCO), and 100 U/ml PenStrep. CSC-enriched populations of cells were obtained by plating a single cell suspension of breast, ovary, and prostate tumour cells at 10000 cells/cm² in SFM on Low-adherent six-well plates (Corning). CSCs were maintained in SFM for 3 days. The spheroids were dissociated and maintained in CSC culture medium on low-attachment-surface plates for functional studies.

3.2.3 Peptide Design

The peptides were designed from the overlapping sequences of sFRP4 domains and synthesised by Imgenex India Pvt. Ltd. Bhubaneshwar, India. Twelve overlapping sequences from the cysteine-rich domain (CRD/FZ) and 8 sequences from the Netrin-like domain (NLD) of sFRP4 were chosen. We have published data using CRD peptides previously (21). Based on extensive preliminary studies, we selected the two most potent peptides i.e. SC301 (CRD derived) and SC401 (NLD derived) from each domain. The details of the peptide sequences cannot be revealed at this point in time due to patent restrictions.

3.2.4 Chemo-sensitisation/Drug treatment

CSC sensitisation with SC301/SC401 was performed by adding peptides to the cell culture at 250 pg/ml for 24 h at 37°C in 5% CO_2 incubator. Doxorubicin (Sigma) was tested at an IC50 value of 1 - 10 µM. Cisplatin (Sigma) was tested at its IC50 value of 10 – 50 µM. The drug treatment for the downstream analysis was optimized at 250 pg/ml of SC301/SC401 alone or in combination with 5 µM of doxorubicin (breast tumour cells only) and 30 µM of cisplatin (ovary and prostate tumour cells only) for 24 hr. An MTT assay was used for the analysis of cellular viability.

3.2.5 Cell Surface Markers

To assist in determining the cells' identity, the expression of cell surface markers was examined in CSCs by flow cytometry (BD FACSCANTO II), as previously published (11).

3.2.6 Cell Viability Assay (MTT)

Cell viability kit - an MTT kit (Sigma) was used to measure cell metabolic viability.

Monolayers - 5000 cells/cm² were plated in a flat-bottomed 96-well plate for 2 days with culture medium. After that, SC301/SC401 alone or in combination with the tumour-specific drug was added and the cells were incubated for 24 h. After drug treatment, the MTT assay was performed as per the manufacturer's instructions.

CSCs - 10000 cells/cm² of monolayer cells were plated in a low-adherent flat-bottomed 96-well plate for 3 days in non-adherent SFM conditions. After that, SC301/SC401 alone or in combination with the tumour-specific drug treatment was added and the cells left for 24 h, following which the MTT assay was performed. Plates were read at 595 nm using an EnSpire Multilabel Plate Reader (Perkin-Elmer).

3.2.7 Mitochondrial depolarisation (an indicator of apoptosis)

JC-1 Assay: JC-1 enters the mitochondria and changes its fluorescent properties based on aggregation of the probe, and forms complexes known as J-aggregates with intense red fluorescence. JC-1 activity was measured using the JC-1 Mitochondrial Membrane Potential Assay Kit (Cayman, USA). CSCs were enriched in 96 well low attachment plates, and treated with SC301/SC401 alone and in combination with chemotherapeutic drugs for 24 h and compared with untreated CSCs. Post-treatment, 10µl of JC-1 staining solution was added to each well and incubated at 37°C for 30 mins. The plate was washed with assay buffer at 400g for 5 mins twice. This was followed by addition of 100µl of assay buffer to each well and read using an EnSpire Multilabel Plate Reader (Perkin-Elmer) for analysis. JC-1 aggregates were measured at excitation and emission wavelengths of 535nm and 595nm respectively. JC-1 monomers were measured at excitation and emission wavelengths of 485nm and 535nm. The ratio of fluorescent intensity of J-aggregates and J-monomers (Red: Green) was used as an indicator of mitochondrial depolarisation and indirectly, cell health.

The JC-1 assay kit is highly light sensitive and all procedures were conducted in dark conditions.

Apoptosis determination (Caspase-3 Assay): Caspase-3 activity was measured using the EnzChek Caspase-3 Assay Kit II (Molecular Probes, Invitrogen). Briefly, 50 µl of the supernatant was added to an individual well of a 96-well micro fluorescent plate and incubated for 10 min at room temperature. After incubation, 50 µl of the 2x working substrate (5 M Z-DEVD-R110) were added to each well and further incubated for 30 min at 37°C. Fluorescence was measured at 485 nm excitation and 538 nm emissions using an EnSpire Multilabel Plate Reader (Perkin-Elmer). Caspase-3 activity was expressed as arbitrary units of fluorescence.

3.2.8 Western blot analysis

Cells were washed twice with PBS and then lysed in RIPA lysis buffer, Sigma (150 mM NaCl, 1.0% IGEPAL® CA-630, 0.5% sodium deoxycholate, 0.1% SDS, 50 mM Tris, pH 8.0, Proteinase Inhibitor 1x). Post-sonication, cell lysates were centrifuged at 14000g for 10 min at 4 °C, and the supernatants were used for Western blotting. The lysates were resolved by sodium dodecyl sulphate-polyacrylamide gel electrophoresis, transferred onto nitrocellulose membranes, and then stained with 0.1% Ponceau S solution (Sigma-Aldrich, St Louis, MO, USA) to ensure equal loading of the samples. After being blocked with 5% non-fat milk for 60 min, the membranes were incubated with primary antibodies ABCG2 (Cell Signaling #42078); c-Myc (Cell Signaling #5605); Cyclin D1 (Abcam #ab134175); Bcl-xL (Cell Signaling #2764); Bax (Cell Signaling #5023); β-Actin (Cell Signaling #4970) overnight, and the bound antibodies were visualized with horseradish peroxidase-conjugated secondary antibodies using the ECL Western Blotting Substrate (Amersham, GE) on a Chemi-Doc (Bio-Rad) imaging analyzer. The primary antibodies concentrations are described in Supplementary data in our previously published study (11).

3.3 Statistics

Statistical analysis was performed with GraphPad Prism V5.0 (GraphPad software, La Jolla, USA) using one-way ANOVA for analysis variance with Bonferroni test for comparison, showing significance as ***P<0.001; **P<0.01; *P<0.05. Data are presented as mean ± standard error of the mean.

3.4 Results

3.4.1 Peptides (SC301/SC401) in combination with Doxorubicin/Cisplatin reduce the sphere forming capacity of CSCs.

The CSCs derived from breast, prostate, and ovary tumour cell lines were treated with SC301/SC401 (250 pg/ml) and Doxorubicin (5µM)/Cisplatin (30µM) alone or in combination. The untreated spheroids remained intact, whereas the combinatorial treatment of SC301/SC401 and chemotherapeutic drugs showed disruption of spheroids post-treatment (Figure 1), indicating the peptides' capacity to segregate the tumour spheres and allow chemotherapeutic drugs to inhibit tumour cell aggregation and proliferation. The peptides alone also showed sphere disruption, indicating their capacity to inhibit spheroid formation.

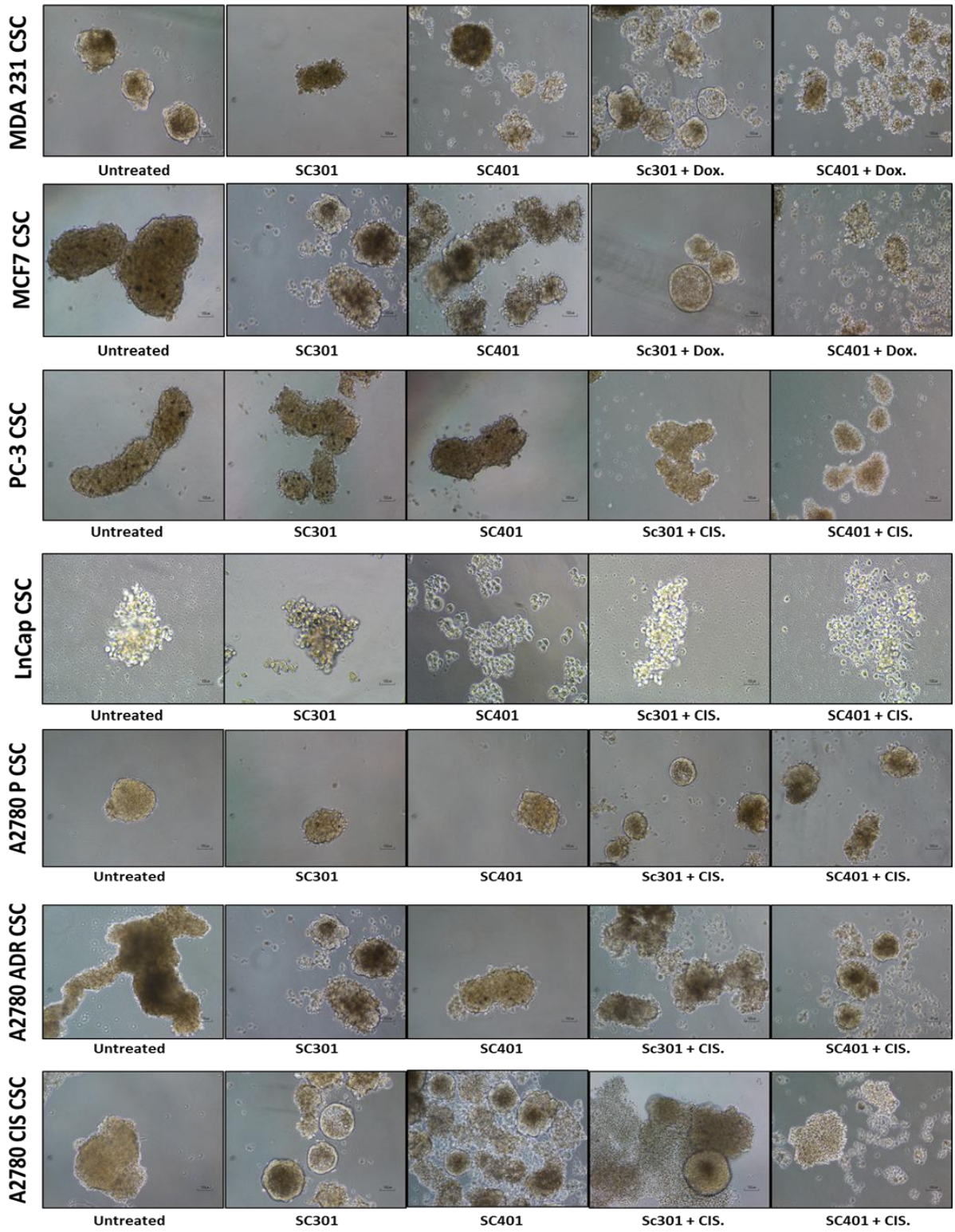


Figure 1: Effect of peptides on CSC morphology: CSCs were isolated from breast, ovary, and prostate tumour cell lines and treated with peptides SC301 (250pg/ ml) and SC401 (250pg/ ml) with chemotherapeutic drugs (Doxorubicin 5µM/Cisplatin 30µM). The combinatorial treatment shows the disruption of the CSC spheres. (Scale bar: 100 µm). Cells images are representative from all the experiments.

3.4.2 Tumour derived CSC characterization

Spheroids obtained for CSC isolation were characterized for the expression of tumour-specific CSC markers $CD44^+$ $CD24^-$/low for breast CSCs, and $CD133^+$/ $CD44^+$ for prostate and ovarian CSCs (Figure 2), by using flow cytometry. The combinatorial treatment demonstrated a significant reduction in the CSC marker population in all cell line-derived CSCs; although in A2780 prostate CSCs, Cisplatin treatment demonstrated phenotype switching to $CD44^+$ positive cells and only reduced the $CD133^+$ population; however, this switching did not affect the inhibitory effect of combinatorial treatment. The characterized CSCs were further used for functional analysis. The effects of treatment with Doxorubicin and Cisplatin alone on these cell lines, and the no treatment data, have been previously reported in our earlier study and have not been shown here (11).

CSC Markers / CSC Treatments	$CD44^+/ CD24^-$ (%)		$CD133^+/ CD44^+$ (%)				
	MDA231	MCF-7	PC-3	LnCap	A2780 P	A2780 CIS	A2780 ADR
Untreated	58.1	36.73	24.3	62.7	2.85	19.5	2.72
Dox/ Cis	28.50	14.9	22.1	47.17	9.29	16	1.8
SC301	47.40	25.27	20.40	58.43	2.37	18.20	2.47
SC401	44.50	29.30	20.33	46.77	2.13	17.57	2.38
SC301 + Dox/ Cis	25.43	13.47	16.48	37.37	1.70	14.97	1.53
SC401 + Dox/ Cis.	21.23	13.80	17.43	31.83	1.40	13.83	1.35

Figure 2: Effect of peptides on CSC surface markers: Using flow cytometry, CSCs were characterized using $CD44^+/ CD24^{-/low}$ for breast CSCs and $CD133^+/ CD44^+$ for prostate and ovary CSCs. The combinatorial treatment shows a reduction in CSC surface markers. Statistical analysis of CSC markers post-treatment. Data are cumulative means ± percentage from 3 independent experiments.

3.4.3 Peptides (SC301/SC401) in combination with Doxorubicin/Cisplatin reduce CSC viability

Using an MTT assay, it was observed that the combinatorial treatment of SC301/SC401 and Doxorubicin/Cisplatin significantly inhibited the viability of CSCs (P<0.001, n=3) compared to peptides or drugs alone. Similar patterns were observed in all three cell lines (Figure 3). Therefore, this treatment combination was used for subsequent studies.

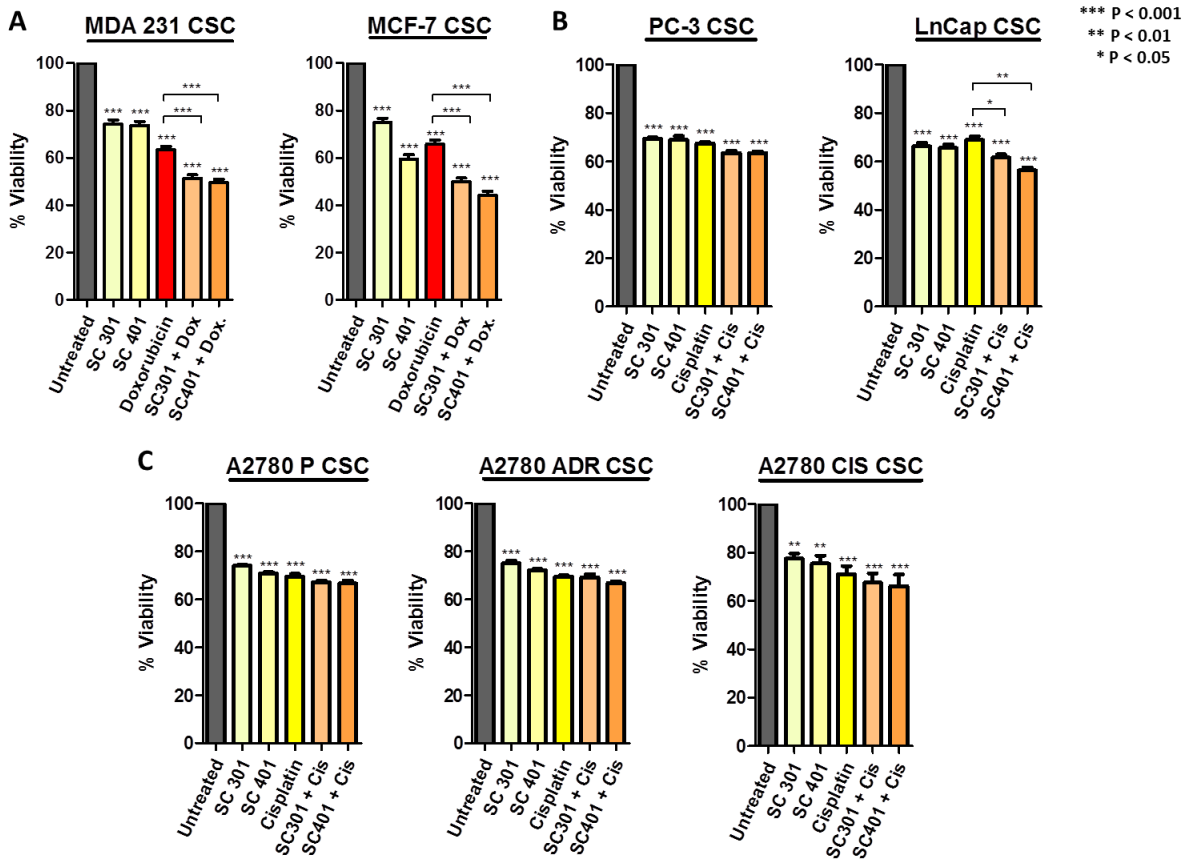


Figure 3: Effect of peptides on CSC viability: A viability assay was performed by MTT after treatment of CSCs derived from A) Breast tumour cell lines (MDA231/ MCF7). B) Prostate tumour cell lines (PC3, LnCap). C) Ovary tumour cell lines (A2780 P, CIS, ADR) with peptides alone or in combination with Doxorubicin/Cisplatin for 24 hr. Statistical analysis was performed using ANOVA for analysis variance with Bonferroni test for comparison showing significance as ***P<0.001; **P<0.01; *P<0.05. Data are mean ± standard error of the mean from 3 independent experiments.

3.4.4 Peptides (SC301/SC401) mediate early apoptotic events in CSCs

The disruption of mitochondrial membrane potential was investigated by using the JC-1 dye. Results from the JC-1 assay demonstrated a significant increase ($p<0.01$) in mitochondrial depolarization after treatment with SC301/SC401, Doxorubicin/Cisplatin alone, and in combinatorial treatments when compared to untreated control. In all cell line-derived CSCs, maximum depolarization was observed with combinatorial treatments, indicating early stage death and apoptotic response through SC301/SC401 (Figure 4). To further confirm the apoptotic effect of peptides on CSCs, we studied caspase-3 activity in CSCs derived from all cell lines, which indicated increased caspase-3 activity ($p<0.001$) in the SC301/SC401 alone and combinatorial treatments in comparison to untreated cells (Figure 5).

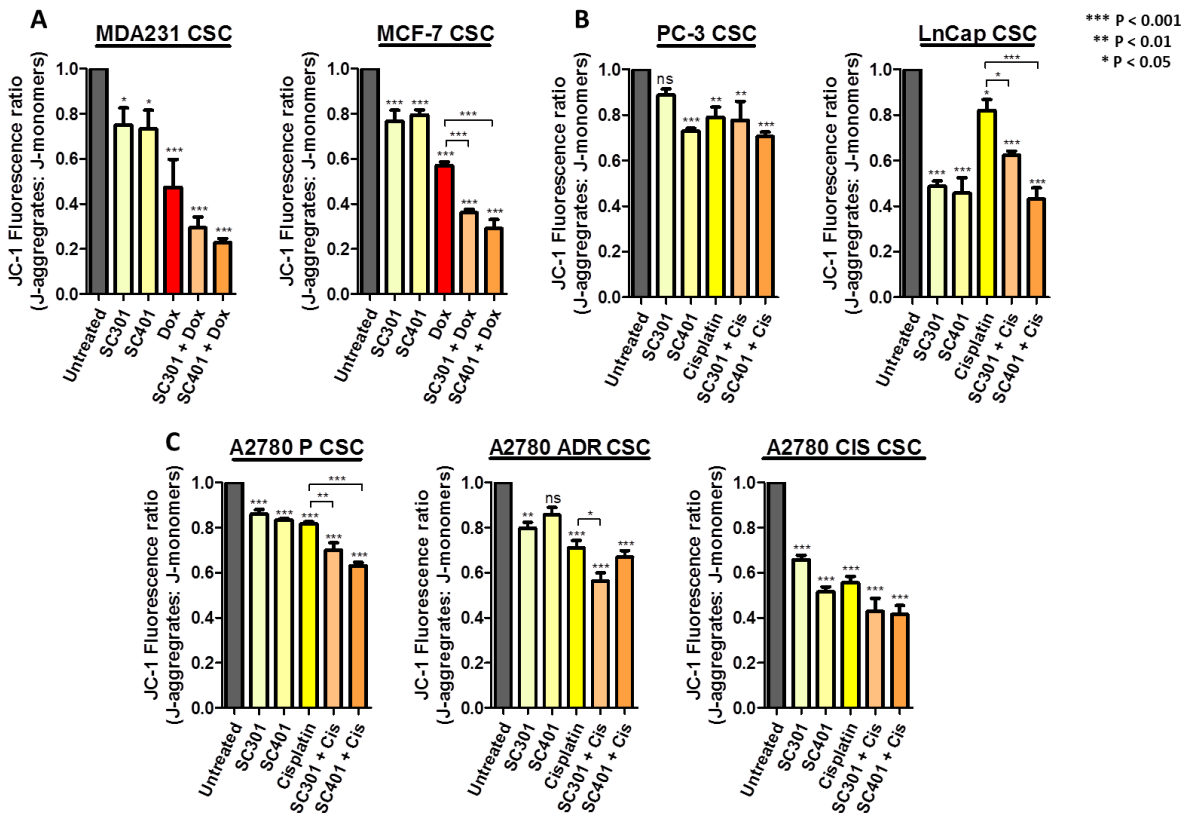


Figure 4: Peptides disrupt mitochondrial membrane potential: Detection of JC-1: The JC-1 assay demonstrated a high mitochondrial depolarization in CSCs derived from A) Breast tumour cell lines (MDA231, MCF7). B) Prostate tumour cell lines (PC3, LnCap). C) Ovary tumor cell lines (A2780 P, CIS, ADR). Treatment with peptides (SC301/SC401), Doxorubicin/Cisplatin, and their combination, indicating early stage cell death and apoptotic response to the various drug treatments. The combinatorial treatment on CSCs showed maximum depolarization. Statistical analysis was performed using ANOVA for analysis variance with Bonferroni test for comparison showing significance as ***P<0.001; **P<0.01; *P<0.05. Data are mean ± standard error of the mean from 4 independent experiments.

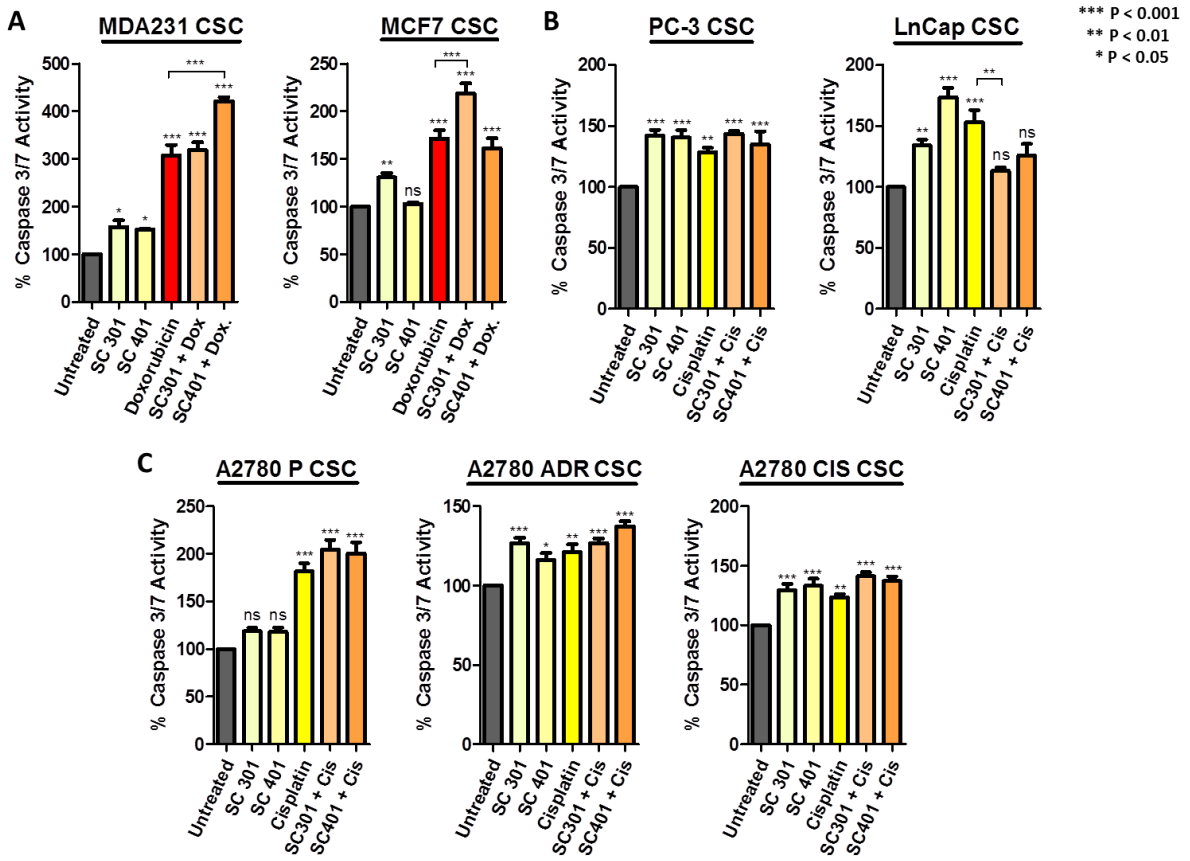
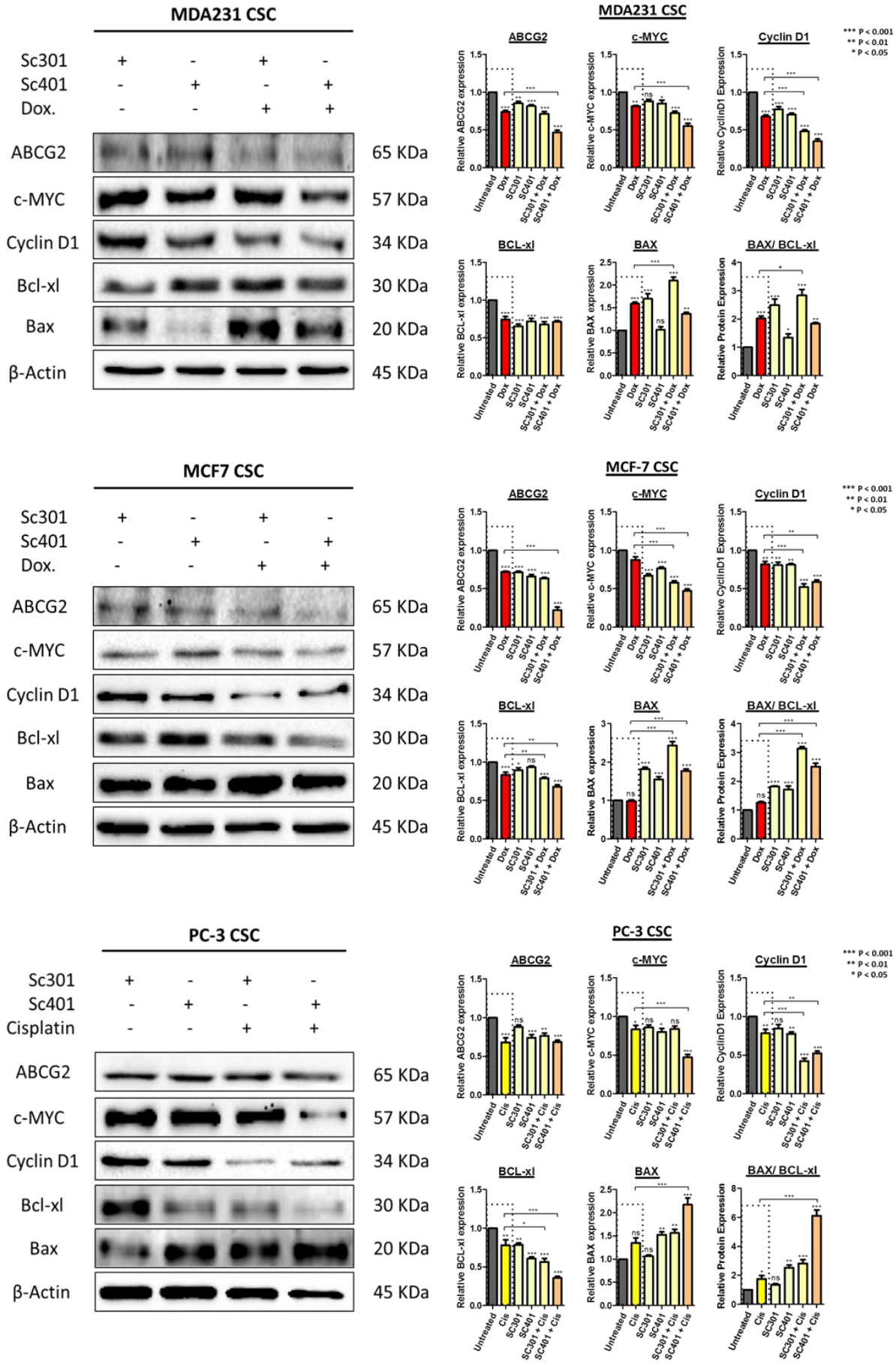
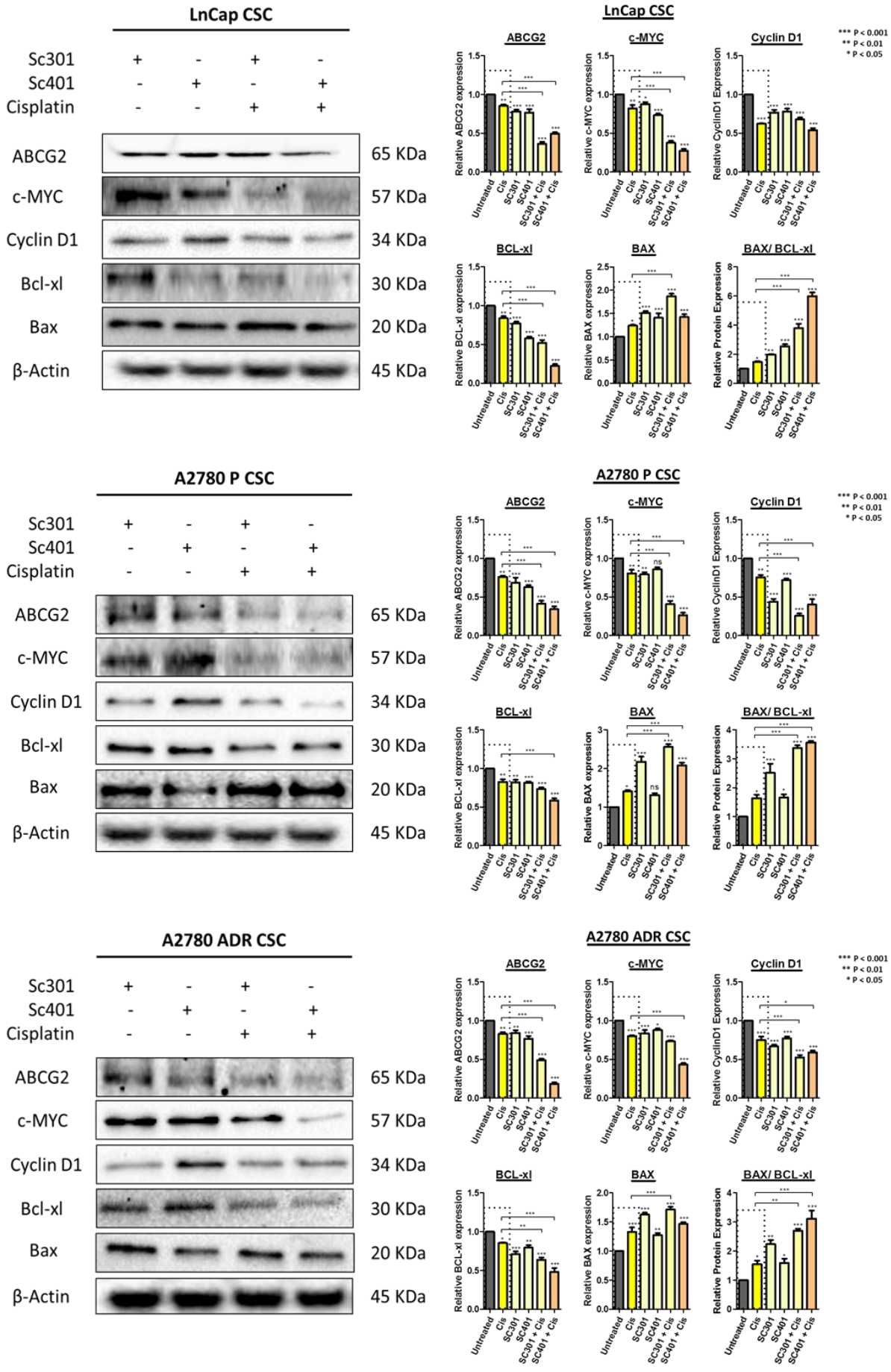


Figure 5: Peptides initiate apoptotic events in CSCs: Increasing amount of caspase-3 substrate indicates initiation of apoptosis. The caspase-3 activity (an indicator of late apoptosis) of CSCs derived from A) Breast tumour cell lines (MDA231, MCF7). B) Prostate tumour cell lines (PC3, LnCap). C) Ovary tumour cell lines (A2780 P, CIS, ADR) was significantly upregulated in combinatorial treatment compared to untreated cells. Statistical analysis was performed using ANOVA for analysis variance with Bonferroni test for comparison showing significance as ***P<0.001; **P<0.01; *P<0.05. Data are mean ± standard error of the mean from 4 independent experiments.

3.4.5 Peptides (SC301/SC401) regulate protein expression in CSCs

Following SC301/SC401 treatment, we investigated the post-translational modifications in CSCs for ABC transporters (ABCG2), oncogenes (c-Myc), anchorage independent cell survival (Cyclin D1), anti-apoptotic (Bcl-xl), and pro-apoptotic proteins. ABCG2 was highly expressed in the untreated groups but decreased in the presence of SC301/SC401, Doxorubicin/Cisplatin, and combinatorial treatments, with the latter inducing the lowest expression levels. SC401 decreased ABCG2 significantly in all CSCs, except in PC-3 CSCs (Figure 6C). The SC301 decreased expression levels of c-MYC in all CSCs, but SC401 combinatorial treatment decreased c-MYC significantly in all CSCs compared to SC301. The level of the proto-oncogene cyclin D1 was decreased in all the combinatorial treatments of CSCs compared to untreated CSCs, except in prostate LnCap CSCs (Figure 6D). Over-expression of the anti-apoptotic protein Bcl-xl in untreated CSCs confers chemo-resistance; however, the combinatorial treatment produced a significant decrease in protein expression levels, indicating SC301/SC401 peptides' pro-apoptotic capacity. Expression of pro-apoptotic protein Bax was lower in untreated CSCs but increased significantly with combinatorial treatment. The increased Bax/Bcl-xl expression level ratio confirms the pro-apoptotic role of SC301/SC401. The effects of treatment with Doxorubicin and Cisplatin alone on these cell lines, and the no treatment data, have been previously reported in our earlier study and have not been shown here (11).


MDA231 CSC
Sc301 + - + -
Sc401 - + - +
Dox. - - + +
ABCG2 65 KDa
c-MYC 57 KDa
Cyclin D1 34 KDa
Bcl-xl 30 KDa
Bax 20 KDa
β-Actin 45 KDa
MDA231 CSC
ABCG2
c-MYC
Cyclin D1
BCL-xl
BAX
BAX/ BCL-xl
Relative ABCG2 expression
Relative c-MYC expression
Relative CyclinD1 Expression
Relative BCL-xl expression
Relative BAX expression
Relative Protein Expression
Untreated
Dox
SC301
SC401
SC301 + Dox
SC401 + Dox
*** P < 0.001
** P < 0.01
* P < 0.05
MCF7 CSC
Sc301 + - + -
Sc401 - + - +
Dox. - - + +
ABCG2 65 KDa
c-MYC 57 KDa
Cyclin D1 34 KDa
Bcl-xl 30 KDa
Bax 20 KDa
β-Actin 45 KDa
MCF-7 CSC
ABCG2
c-MYC
Cyclin D1
BCL-xl
BAX
BAX/ BCL-xl
Relative ABCG2 expression
Relative c-MYC expression
Relative CyclinD1 Expression
Relative BCL-xl expression
Relative BAX expression
Relative Protein Expression
Untreated
Dox
SC301
SC401
SC301 + Dox
SC401 + Dox
*** P < 0.001
** P < 0.01
* P < 0.05
PC-3 CSC
Sc301 + - + -
Sc401 - + - +
Cisplatin - - + +
ABCG2 65 KDa
c-MYC 57 KDa
Cyclin D1 34 KDa
Bcl-xl 30 KDa
Bax 20 KDa
β-Actin 45 KDa
PC-3 CSC
ABCG2
c-MYC
Cyclin D1
BCL-xl
BAX
BAX/ BCL-xl
Relative ABCG2 expression
Relative c-MYC expression
Relative CyclinD1 Expression
Relative BCL-xl expression
Relative BAX expression
Relative Protein Expression
Untreated
Cis
SC301
SC401
SC301 + Cis
SC401 + Cis
*** P < 0.001
** P < 0.01
* P < 0.05


LnCap CSC
Sc301 + - + -
Sc401 - + - +
Cisplatin - - + +
ABCG2
c-MYC
Cyclin D1
Bcl-xl
Bax
β-Actin
65 KDa
57 KDa
34 KDa
30 KDa
20 KDa
45 KDa
ABCG2
c-MYC
Cyclin D1
BCL-xl
BAX
BAX/ BCL-xl
Relative ABCG2 expression
Relative c-MYC expression
Relative CyclinD1 Expression
Relative BCL-xl expression
Relative BAX expression
Relative Protein Expression
Untreated
Cis
SC301
SC401
SC301 + Cis
SC401 + Cis
*** P < 0.001
** P < 0.01
* P < 0.05
A2780 P CSC
A2780 ADR CSC

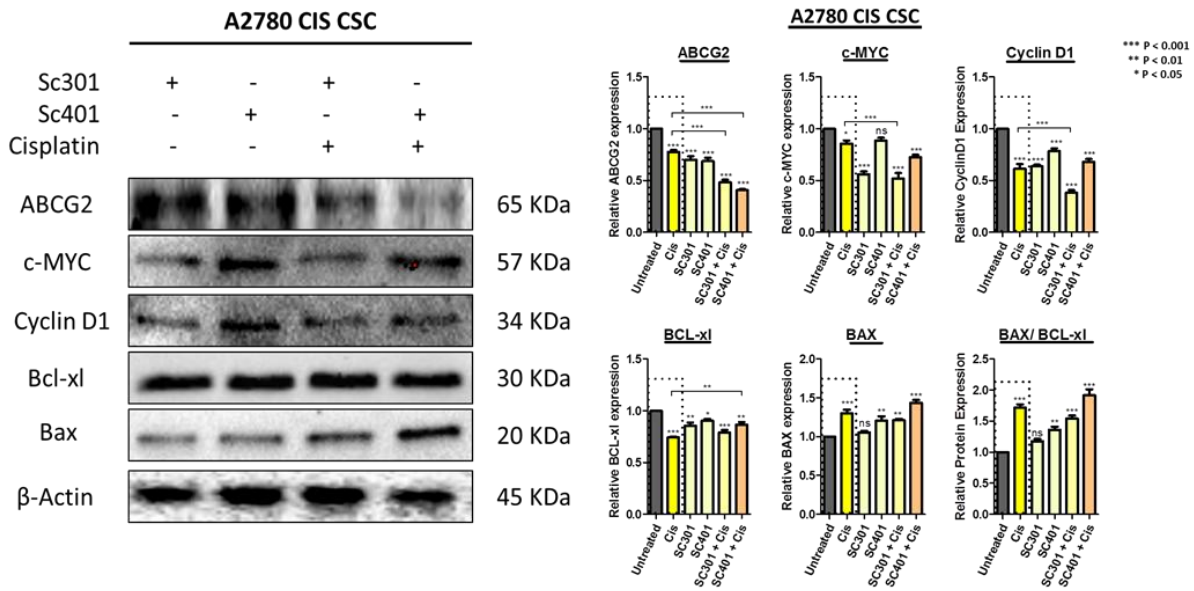


Figure 6: Effects of peptides on protein expression levels: The combinatorial treatment of SC301/SC401 and chemotherapeutic drugs significantly increased the apoptotic protein (22) and decreased the drug efflux protein (ABCG2), cell survival (Cyclin D1), and oncogene (C-Myc) expression. A) Breast MDA-MB 231 CSCs; B) Breast MCF7 CSCs; C) Prostate PC3 CSCs; D) Prostate LnCap CSCs; E) Ovary A2780 Parental CSCs; F) Ovary A2780 Adriamycin-resistant CSCs; G) Ovary A2780 Cis-Resistant CSCs. In the combinatorial treatment, an elevated BAX/Bcl-xl ratio corresponded to elevated cell apoptosis. Statistical analysis was performed using ANOVA for analysis variance with Bonferroni test for comparison showing significance as ***P<0.001; **P<0.01; *P<0.05. Blots and relative protein expressions are mean ± standard error of the mean from 3 independent experiments.

3.5 Discussion

Resistance to chemotherapeutic drugs is one of the major barriers towards effective cancer therapy. Increasing evidence suggests that chemotherapy failure is due to the presence of CSCs, which rely on regulatory pathways such as Wnt, Notch, and Hedgehog to maintain their self-renewal and differentiation potency (23). However, aberrant β-catenin signalling and ABCG2 expression are closely related to CSCs' chemo-response (20, 24). Recent studies have identified seven functional transcription factors with binding sites in the ABCG2 gene promotor, indicating ABCG2 might be a downstream gene of β-catenin (25). Therefore, targeting aberrant Wnt/β-catenin signalling has become an important approach to overcome chemotherapy failure. This study presents two important findings. First, peptides derived from sFRP4 (SC301\SC401) inhibit CSC survival. Second, these peptides enhance Doxorubicin and Cisplatin anti-cancer effects. These effects indicate inhibition of the Wnt/β-catenin signalling pathway by these sFRP4-derived peptides. Our study is the first to report the role of these novel peptides on chemo-sensitisation of breast, prostate, and ovarian CSCs. Our study demonstrates that the peptides either alone or in combinatorial treatment with chemotherapeutic drugs elicit anti-proliferative effects, spheroid disruption, decrease in cell survival, and initiation of apoptosis within the CSCs, therefore indicating chemo-sensitization.

CSCs from tumour cell lines (breast – MDA231, MCF7; prostrate - PC3, LnCap; ovary – A2780 P, A2780 ADR, A2780 CIS) were selected based on the following features: The ability to form spheroids in serum free conditions; increased expression of CSC surface markers, elevated expression of ABC drug transporters (ABCG2), cell survival protein (Cyclin D1), oncogene (c-Myc) and the ability to escape cell death/apoptosis (Bcl-xl). Resistance of CSCs to chemotherapeutic drugs is often found to be in correlation to CSC surface marker profiles, such as seen in $CD44^{+high}$ and $CD133^{+high}$ cells that are highly radio-resistant in colon cancer and have shown high DNA repair capacity and increased evasiveness to apoptosis in comparison to cells low in the CD44 and CD133 surface markers (26). In this study, reduction in the expression of CSC markers was observed and it was found that SC301/SC401 was able to decrease the $CD44^{+}$ 24^{-} population of breast-derived CSCs and the $CD44^{+}$ $CD133^{+}$ population of prostrate and ovary-derived cells when tested in combination with chemotherapeutic drugs. Previously, it was shown that cisplatin treatment in ovarian A2780

P derived CSCs aberrantly increased the $CD133^+$ $CD44^+$ populations when together and decreased $CD44^+$ and $CD133^+$ when alone and this aberration implies phenotypic switching of CSCs through a process that is still not well characterized (27).

Preservation of CSCs' phenotypic plasticity, facilitation of metastatic potential and the increased expression of drug efflux transporters make CSCs highly chemo-resistant. This is facilitated by CSCs residing in anatomically distinct regions ('niches') within the tumour microenvironment (28). Isolation and characterization of CSCs from *in-vitro* CSC niches have shown spheroid forming capacity in serum free conditions (29). Previously, our studies demonstrated that the niches can be disrupted by targeting the Wnt signalling pathway using sFRP4 in combination with chemotherapeutic drugs (11). The disruption of spheroids by SC301 and SC401 was shown to decrease CSC plasticity and cell-cell adhesion, which led to the initiation of CSC differentiation towards a tumour cell phenotype and reduced their capacity for self-renewal. This demonstrated the high potency of using chemotherapeutic drugs to target cells and has shown that SC301 and SC401 decreased the viability of CSCs when used in combination with chemotherapeutic drugs rather than through drug treatment alone. This indicates the potential of these peptides for an increased chemo-response of CSCs.

Alterations in expression of anti-apoptotic (Bcl-2 family) and pro-apoptotic genes have previously been shown to result in chemo-resistance of CSCs (30). There are two pathways that trigger apoptosis: 1) initiation of caspase-9 activation by the ligand of death receptors on the cell surface (extrinsic pathway); 2) Release of cytochrome c by mitochondria (intrinsic pathway) (31). The downstream executioners caspase-3/7 are activated by the assembly of the apoptosome by caspase-9, which is activated in a crucial step by the release of cytochrome c (32). The relationship between sFRP4 and apoptosis has been studied previously and shown that sFRP4 acts as a pro-apoptotic agent (33, 34). In this study, mitochondrial depolarization was tested by using the JC1-assay. Apoptosis was indicated by low mitochondrial membrane potential $\Delta\psi M$ as in mitochondrial depolarization, thereby indicating that the involvement of peptides SC301 and SC401 in the initiation of apoptotic pathways. Furthermore, the pro-apoptotic role of the peptides and later onset of apoptosis in CSCs treated with SC301 and SC401, either alone or in combination with

chemotherapeutic drugs, was demonstrated by the elevation in expression of caspase-3/7 in the CSCs treated with these peptides.

The Bax/Bcl-xl ratio is used as an indicator for apoptosis, with an increased ratio depicting the activation of caspase-3 (35). We hypothesized that the peptides SC301 and SC401 had the potential to bind the hydrophobic groove of apoptotic Bcl-2, which would oligomerize Bax and subsequently lead to mitochondrial membrane potential depolarization and the subsequent release of cytochrome c. An increase in Bax expression and a decrease in Bcl-xl expression was demonstrated when SC301 and SC401 were used either alone or in combination. Furthermore, the Bax/Bcl-xl ratio was found to be consistently high in all the CSCs that were subjected to combinatorial treatment. The role of the peptides as a pro-apoptotic agent was also indicated by the elevated expression of apoptotic genes within all the CSCs used in the study.

A decrease in the expression of the oncogene that drives cell cycle progression, Cyclin D1, was observed. Cyclin D1 has known roles in interaction of proteins involved in DNA repair, RNA metabolism and cell structure and its deregulation affects cellular processes and leads to inefficient DNA damage repair systems (36). Wnt signalling activates the expression of GSK-3β, which in turn regulates Cyclin D1 expression in the Wnt signalling pathway (37, 38). Being a Wnt antagonist sFRP4 binds to the frizzled receptors and activates the GSK-3β destruction complex resulting in the initiation of Cyclin D1 degradation.

Furthermore, it was found that SC401 (either alone or in combinatorial treatment) results in the decrease of expression of c-Myc, onco-protein with important regulatory function in stem cell biology that correlates to tumour metastasis (39, 40). CSCs exhibit a high expression of c-Myc, therefore the downregulation of this onco-protein result in apoptosis under various conditions (41-44). We hypothesized the targeted reduction in c-Myc expression was due to the target specificity of SC401 and SC301.

In summary, the results of this study have demonstrated that sFRP4 derived peptides, SC301 and SC401, are able to specifically target CSCs derived from breast, prostate and ovary tumour cell lines by their ability to inhibit Wnt/ β-catenin signalling. Target specificity of these peptides is suggested by their ability to interfere in different cellular pathways in CSCs. This opens the therapeutic window for chemotherapy without affecting normal Wnt-

dependent tissues. The study, thus, highlights the potential of these novel peptides in reduction of chemotherapeutic load, prevention of tumour relapse and ultimately, tumour resolution.

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Author contributions

AbhiD drafted the outline and generated the data. SK contributed the viability data. AbhiD wrote the manuscript. AbhiD and ArunD conceived the study, and ArunD, FA, and PN critically reviewed, revised, and approved the final manuscript.

Declaration of interests

The authors declare that they have no competing interests.

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Chapter 4

Deshmukh, A., Arfuso, F., Newsholme, P., & Dharmarajan, A. (2017). Epigenetic demethylation of sFRPs with emphasis on sFRP4 activation leading to Wnt signalling suppression and histone modifications in breast, prostate, and ovarian cancer stem cells.

(Manuscript is submitted and under revision)

4 Epigenetic demethylation of sFRPs with emphasis on sFRP4 activation leading to Wnt signalling suppression and histone modifications in breast, prostate, and ovarian cancer stem cells.

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Keywords

Cancer stem cells; Epigenetics; Wnt signalling pathways; Secreted fizzled-related protein; DNA methylation; Histone modification.

Abbreviations

CSCs: Cancer Stem Cells; PcG: Polycomb gene; PRC: Polycomb repressive complex; CpG: 5'-C-phosphate-G-3'; DNMT: DNA methyltransferase; EZH2: Enhancer Zeste Homologue 2; MSP: Methylation specific PCR; HDAC: Histone deacetylase; HMT: Histone methyltransferases; 5-Aza: 5 Azacytidine; PTM: Post-translational modification; sFRPs: Secreted frizzled-related proteins; H3K27me3: Tri methyl Histone H3 lysine 27.

Abstract

The expression levels of secreted frizzled-related proteins (sFRPs), important Wnt signalling antagonists, have been reported to be reduced in various cancers, and are associated with disease progression and poor prognosis. During tumour development, all *sFRP (1, 2, 3, 4, and 5) genes* are hypermethylated, causing transcriptional silencing. sFRPs have an ability to sensitize tumour cells to chemotherapeutic drugs, enhancing cell death. Reduced Wnt signalling is associated with loss of cancer stem cell (CSC) viability. We investigated the possible involvement of methylation-mediated silencing of the sFRP gene family in CSCs derived from breast, prostate, and ovarian tumour cell lines. Real-time RT-PCR studies indicated that loss or downregulation of *sFRP* (*1-5*) expression in tumours is associated with promoter hypermethylation. Additionally, CSCs derived from all tumour cell lines with *sFRP* (*1-5*) promotor hypermethylation expressed *sFRP* (*1-5*) mRNA after treatment with 5-Aza, especially *sFRP4*, implying that DNA methylation is the predominant epigenetic mechanism for *sFRP* (*1-5*) silencing. Furthermore, post-translational modification (PTM) in total and histone proteins was observed post 5-Aza and sFRP4 treatment. Protein levels of Wnt downstream signalling components (GSK3β, active β-catenin, and phospho β-catenin) and epigenetic factors of histones (EZH2, acetyl histone H3, and H3K27me3) affecting PTM were analysed.

Our findings suggest that downregulation of *sFRP4* expression in endocrine-related cancers can be attributed to aberrant promoter hypermethylation in conjugation with histone modification, and indicate the important role of methylation-induced gene silencing of *sFRP4* in survival and proliferation of CSCs derived from these cancers.

4.1 Introduction

Various factors contribute to the progression of cancer and benefit the alterations of tumour cells. These alterations include cell cycle deregulation, resistance to apoptosis, and aberrant activation of signalling pathways that promote invasiveness, treatment-resistance, and angiogenesis (1). The cellular epigenetic alterations are due to the fundamental changes within the tumour cell epigenome; hence, studying epigenetic modulations in tumour cells could explain their role in the initiation and progression of human cancer (2-4). Mutated epigenetic modulators in cancer are the most common cause leading to oncogenic cellular reprogramming and promoting uncontrolled self-renewal capacity (5). Furthermore, epigenetic modulations such as DNA methylation, histone modifications, and chromatin remodelling result in promoting malignant phenotypes at various stages of cancer. Aberrant epigenetic alterations may transform progenitor cells to different lineages, leading to tissue differentiation (6). More importantly, epigenetic alterations can result in variability in treatment response. It has been demonstrated that a small subpopulation of tumour cells is resistant to chemotherapeutic drugs (7) in a variety of cancers such as breast, ovary, and prostate, which could be due to the aberrant expression of key epigenetic factors (8). This sub-population of tumour cells is referred to as cancer stem cells (CSCs), and they possess stem-like properties with differentiation capacities and high regenerative characteristics (9). The emergence of CSCs requires elaborate epigenome reorganisation, which facilitates the integration of epigenetic mechanisms to establish intra-tumoural heterogeneity (5).

The modulation of epigenetic factors allows targeting downstream signalling pathways, which determine the CSCs' state. The Wnt signalling pathway plays a crucial role in CSC proliferation, differentiation, and self-renewal (10, 11). Loss of negative regulators such as glycogen synthase kinase 3β (GSK3β), and accumulation of β-catenin in the cytoplasm leads to upregulation of gene transcription and enhances tumour development (12-14, 15). Aberrant activation of Wnt signalling inactivates GSK3β, thus enabling increased accumulation of unphosphorylated β-catenin. Accumulated cytoplasmic β-catenin translocates to the nucleus, activating target genes responsible for tumour development and progression (1, 16, 17). Therefore, Wnt antagonists such as the secreted frizzled-related proteins (sFRPs 1-5) play a crucial role in controlling pathway aberrations and thus tumour

development (18). SFRP4 has the ability to chemo-sensitize CSCs from various tumours, reducing their viability (7, 19, 20).

The *sFRPs* are shown to be hypermethylated in various tumours, inducing transcriptional silencing (21, 22). These epigenetic modification of *sFRPs* (22, 23) indicate DNA methylation as an important mechanism to target gene silencing (24). Furthermore, histone modifications such as acetylation, methylation, and phosphorylation can alter gene expression. These alterations influence biological processes such as DNA damage repair and uncoiling of chromatins (25, 26 , 27). Histone modifications and their co-factors are crucial to understand, such as post-translational modification (PTM) that is responsible for accelerating transcription and hindering DNA methylation (28, 29). Thus, histone modifications are involved in various epigenetic processes of tumour development and progression (30).

The objective of this study was to investigate the involvement of methylation-mediated silencing of the *sFRP* gene family and the effect of sFRP4 on histone modifications in CSCs derived from breast, prostate, and ovarian tumour cell lines. To study the hypermethylation/demethylation of DNA, the DNA (cytosine-5)-methyltransferase 1 (*DNMT1*) inhibitor 5-Azacytidine (5-Aza) was used, and CSCs were treated with sFRP4 to examine the PTM effect on histone modification and its co-factors.

4.2 Materials and Methods

4.2.1 Cell Culture

4.2.1.1 Monolayer cell culture

Cell culture plates for adherent cells were purchased from Nunc™ (ThermoFisher Scientific). The human breast tumour cell lines MDA-MB 231 (ER-) and MCF-7 (ER+), human ovarian tumour cell lines A2780-P, A2780-ADR, and A2780-Cis, and human prostate tumour cell lines PC-3 (AR-/PSA-) and LnCap (AR+) were purchased from American Type Culture Collection (ATCC, USA). The cells were cultured in RPMI-1640 medium (Gibco #11875-093) supplemented with 10% foetal bovine serum (Bovogen #SFBS) and 100 U/ml PenStrep (Life Technologies #15070063). All cells were maintained at 37°C in a humid incubator with 5% CO_2.

4.2.1.2 Cancer stem cell isolation

For CSC isolation, culture plates with an ultra-low-attachment surface were purchased from Corning Life Sciences. CSCs were cultured in serum-free medium (SFM) containing basal medium RPMI-1640 + DMEM-HG (HyClone, USA #SH30081.02) supplemented with the growth factors bFGF (20 ng/ml) (ProSpec Bio #cyt-085), EGF (20 ng/ml) (ProSpec Bio #cyt-217), and 1× B27 (Gibco #17504044), and 100 U/ml PenStrep (Life Technologies #15070063). CSC-enriched populations of cells were obtained by plating a single cell suspension of breast, ovary, and prostate tumor cells at 10000 cells/cm² in SFM on Low-adherent six-well plates (Corning #3471). CSCs were isolated in SFM; the spheroids are formed at the 3rd day of plating tumour cells. To analyse the effects of 5-Azacytidine (5-Aza) and sFRP4, cells were cultured in medium supplemented with the compounds.

4.2.2 Drug treatment

The drugs used in this study were 5-Azacytidine (Sigma-Aldrich #A2385) and purified sFRP4 (R&D Systems #1827-SF-025). CSCs were incubated with 30µM 5-Aza for 48 h after a 48 h seeding period since this dose and treatment time provided consistent reduction in cell viability across all cell lines. The dose range of 5-Aza was narrowed based on our previous publication (22). CSC sensitization with sFRP4 was performed by adding sFRP4 to the cell culture at 250 pg/ml (7) for 24 hr at 37°C in a 5% CO_2 incubator.

4.2.3 Viability Assay

A cell counting viability kit (CCK8, Sigma- Aldrich #96992) was used for the quantitation of viable cells. Monolayer cells were plated at a density of 10000 cells/cm² in a low-adherent flat-bottomed 96-well plate (Corning #3474) for 3 days in non-adherent SFM conditions. Wells with drug-free medium were used as a negative control. CSCs were treated with 5-Aza for 48 h, then 10 µl of CCK8 solution was added to each well and incubated at 37°C in a 5% CO_2 incubator for 1 h. Plates were read at 450 nm using an EnSpire Multilabel Plate Reader (Perkin-Elmer).

4.2.4 Cell Surface Markers

To assist in determining the cells' identity, the expression of cell surface markers was examined in CSCs by flow cytometry (BD FACSCANTO II), as previously published (7).

4.2.5 Reverse transcription-polymerase chain reaction

Total RNA was isolated from cells using TRIzol reagent (Life Technologies #15596026) followed by chloroform extraction, isopropanol precipitation, and a 75% (v/v) ethanol wash. RNA samples (1 µg) were reverse-transcribed to cDNA using a High Capacity cDNA kit (Applied Biosystems #4368814). cDNA in 1 µl of the reaction mixture was amplified with PCR Master Mix (Life Technologies #K0171) and 10 µM each of the sense and antisense primers. Quantitative real-time (qPCR) was performed using a CFX Connect Real time System (Bio-Rad). Reaction mixtures consisted of 5 µl of 2× KAPA SYBR Fast (Sigma-Aldrich #KK4601), 0.5 µM of each forward and reverse primer, 1 µl of template, 0.2µM ROX low, and RT-PCR Grade water (Life Technologies #AM9935) adjusted to 10 µl. Each reaction mixture was loaded into 96-well PCR plates and amplified under PCR cycling conditions against 2 housekeeping genes (*18S* and *GAPDH*), followed by a melt-curve analysis. Each cycle consisted of denaturation at 95°C for 10s, annealing at 65°C for 10s and extension at 72°C for 15s and these conditions were maintained constant across 40 cycles. The primer sequences are described in Table 1.

Table 2: qPCR Primer sequences and annealing temperatures

Gene	Primer	PCR amplicon size	Annealing Temperature (°C)
sFRP1	F – 5’ atctctgtgccagcgagttt 3’ R – 5’ aagtggtggctgaggttgtc 3’	202	65
sFRP2	F – 5’ aggacaacgacctttgcatc 3’ R – 5’ ttgctcttggtctccaggat 3’	217	65
sFRP3	F – 5’ aaactgtagaggggcaagca 3’ R – 5’ ggcagccagagctggtatag 3’	227	65
sFRP4	F – 5’ cgatcggtgcaagtgtaaaa 3’ R – 5’ gacttgagttcgagggatgg 3’	181	65
sFRP5	F – 5’ gatgtgctccagtgactttg 3’ R – 5` gcaggggtaggagaacatga 3’	322	65
18S	F – 5’ gcaattattccccatgaacg 3’ R – 5’ gggacttaatcaacgcaagc 3’	68	65
GAPDH	F – 5’ cagaacatcatccctgcatccact 3’ R – 5’ gttgctgttgaagtcacaggagac 3’	258	65

4.2.6 Genomic DNA Isolation

Genomic DNA was extracted from all cell lines using a QIAamp DNA Mini Kit (Qiagen #51304) according to the manufacturer's instructions. DNA integrity and quality were evaluated by spectrophotometric analysis.

4.2.7 Bisulfite Treatment

Briefly, 1 µg of genomic DNA was bisulfite treated with the EpiTect Bisulfite Kit (Qiagen #59104) according to manufacturer's instructions. The methylation status of genomic DNA was analysed using methylation-specific PCR.

4.2.8 Methylation-specific PCR

The methylation status of all 5 sFRP genes in the human cell lines was determined by methylation-specific polymerase chain reaction (MSP). Briefly, 1 µg of genomic DNA was bisulfite-treated with the EpiTect Bisulfite Kit (Qiagen #59104). The bisulphite conversion thermal cycler conditions were in following order, denaturation at 95°C for 5 mins, incubation at 60°C for 25 mins, denaturation at 95°C for 5 mins, incubation at 60°C for 85 mins, denaturation at 95°C for 5 mins, incubation at 60°C for 175 mins, rest at 20°C. Bisulfite-treated DNA was amplified using 10µl of 2x PCR Master Mix (Thermo Fisher Scientific, USA), 0.5µM of each forward and reverse primer, 1µl of template DNA, and RT-PCR Grade water (Life Technologies #AM9935) adjusted to a final volume of 20µl. Each reaction was loaded in PCR tubes and amplified in following thermal cycler conditions, denaturation at 95°C for 30s, annealing at 60-68°C, extension at 72°C for 30s and these conditions were maintained constant for 35 cycles. PCR products were analysed by agarose gel electrophoresis. Previously described primers specific for either methylated or unmethylated DNA are shown in Table 2.

Table 3: Methylation-Specific PCR Primer Sequences and annealing temperature

Gene	Specificity	Primers	Annealing Temperature (°C)
sFRP1	Methylated	F – 5’ gtgtcgcgcgttcgtcgtttcgc 3’ R – 5’ aacgttacccgactccgcgaccg 3’	60
	Unmethylated	F – 5’ gagttagtgttgtgtgtttgttgttttgt 3’ R – 5’ cccaacattacccaactccacaacca 3’	60
sFRP2	Methylated	F – 5’ gggtcggagtttttcggagtcgcgc 3’ R – 5’ ccgctctcttcgctaaatacgactcg 3’	62
	Unmethylated	F – 5’ ttttgggttggagtttttttggagttgtgt 3’ R – 5’ aacccactctcttcactaaatacaactca 3’	66
sFRP3	Methylated	F – 5’ ggagcgggtttttttggcg 3’ R – 5’ gaaccccgaacacccgaaa 3’	65
	Unmethylated	F – 5’ ggagtgggtttttttggtgtttattgt 3’ R – 5’ cccaaaccccaaacaccca	63
sFRP4	Methylated	F – 5’ gggtgatgttatcgtttttgtatcgac 3’ R – 5’ cctcccctaacgtaaactcgaaacg 3’	63
	Unmethylated	F – 5’ gggggtgatgttattgtttttgtattgat 3’ R – 5’ cacctcccctaacataaactcaaaaca 3’	60
sFRP5	Methylated	F – 5’ aagatttggcgttgggcgggacgttc 3’ R – 5’ actccaacccgaacctcgccgtacg 3’	65
	Unmethylated	F – 5’ gtaagatttggtgttgggtgggatgttt 3’ R – 5’ aaaactccaacccaaacctcaccataca 3’	68

4.2.9 Western blotting

Cells were washed twice with PBS and then lysed in RIPA lysis buffer (Sigma #R0278) (150 mM NaCl, 1.0% IGEPAL® CA-630, 0.5% sodium deoxycholate, 0.1% SDS, 50 mM Tris, pH 8.0, Proteinase Inhibitor 1x). Post sonication, cell lysates were centrifuged at 14000g for 10 min at 4 °C, and the supernatants were used for Western blotting. The lysates were resolved by sodium dodecyl sulphate-polyacrylamide gel electrophoresis, transferred onto nitrocellulose membranes, and then stained with 0.1% Ponceau S solution (Sigma #P3504) to ensure equal loading of the samples. After being blocked with 5% non-fat milk for 60 min, the membranes were incubated with primary antibodies sFRP4 (1µg/ml, Abcam #ab32784); GSK3β (27C10) (1:1000, Cell Signaling #9315); Non-phospho (Active) β-Catenin (Ser33/37/Thr41) (D13A1) (1:1000, Cell Signaling #8814); Phospho-β-catenin (Ser33/37/Thr41) (1:1000, Cell Signaling #9561); Histone H3 (D1H2) (1:2000, Cell Signaling #4499); EZH2 (D2C9) (1:1000, Cell Signaling #5246); Acetyl-Histone H3 (Lys9) (C5B11) (1:1000, Cell Signaling #9649); Tri-Methyl-Histone H3 (Lys27) (C36B11) (1:1000, Cell Signaling #9733), and β-Actin (13E5) (1:1000, Cell Signaling #4970) overnight at 4°C, and the bound antibodies were visualized with horseradish peroxidase-conjugated secondary antibodies using the ECL Western Blotting Substrate (Amersham, GE #RPN2106) on a Chemi-Doc (Bio-Rad) imaging analyser.

To study the effect of sFRP4 on post-translational modification in histones, CSCs were treated with sodium butyrate (Sigma-Aldrich #B5887) to inhibit histone deacetylase (HDAC) and retain acetylation levels.

4.3 Statistics

Densitometry analysis of Western blots done using Image Lab 4.1 version. Statistical analysis was performed with GraphPad Prism V5.0 (GraphPad software, La Jolla, USA) using two-way ANOVA for analysis variance with Bonferroni test for comparison showing significance as ***P<0.001; **P<0.01; *P<0.05. Data are presented as mean ± standard error of the mean.

4.4 Results

4.5 Increasing 5-Azacytidine concentration reduces CSC viability.

Using a CCK-8 viability assay, it was observed that a high dose (60µM) of 5-Aza at 48 h significantly inhibited the viability of CSCs compared to a lower dose (30µM). The viability inhibition between 30µM and 45µM 5-Aza treated CSCs was significantly less compared to the higher dose of 60µM 5-Aza (Figure 1). Similar patterns were observed in CSCs derived from all the other cell lines. Therefore, to produce less cytotoxic stress on CSCs, while maintaining an equivalent inhibition potency, we decided to select a lower dose of 30µM 5-Aza for CSC treatment in subsequent experiments.

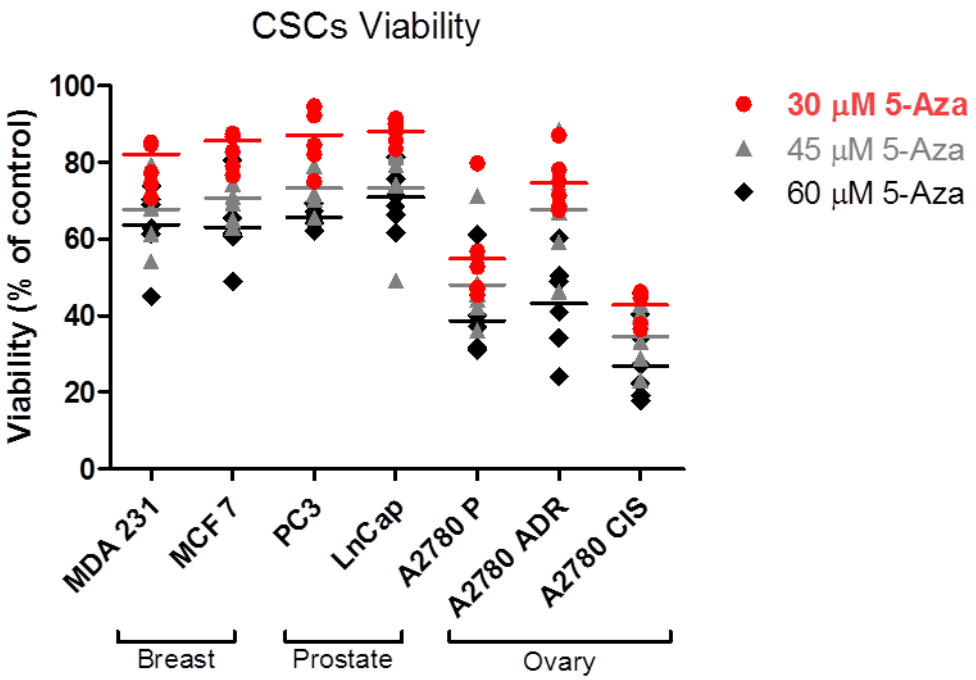


Figure 1: Effect of 5-Azacytidine on viability: The CCK8 viability assay was performed after treatment of CSCs derived from breast (MDA231 and MCF7), prostate (PC3 and LnCap), and ovary (A2780 P, A2780 ADR, and A2780 CIS) tumour cell lines with 5-Aza for 48 h. Statistical analysis was performed using ANOVA for analysis variance with Bonferroni test for comparison. Data are mean ± standard error of the mean from 3 independent experiments.

4.5.1 DNMT1 inhibitor 5-Aza demethylates sFRPs' (1-5) promoter *in vitro*.

To investigate whether there was a direct association of *sFRP* promoter demethylation with increased *sFRP*-specific mRNA expression, we treated CSCs derived from all cell lines with 30μM 5-Aza (Figure 2). Using real time PCR, we determined the *sFRP* promoter methylation before and after drug treatment. The mRNA relative expression post-treatment indicated the *sFRP* promoter demethylation, showing elevated *sFRPs* (*1-5*) expression in most of the tumour-specific CSCs. The *sFRP1* mRNA relative expression was significantly upregulated in all the cell lines, suggesting demethylation post 5-Aza treatment, though A2780 Cis CSC was not statistically significant (Figure 2A). The *sFRP2* mRNA relative expression was upregulated in MDA231 CSCs, A2780 P CSCs, and A2780 Cis CSCs post treatment, but other CSCs did not undergo significant change (Figure 2B). The *sFRP3* mRNA expressions were upregulated in 5 out of 7 CSCs derived from all the cell lines, with the exception of MCF7 and A2780 Cis CSCs (Figure 2C).

Demethylation of its promoter had a functional effect on relative expressions of *sFRP4* specific mRNA. Upon treatment with 5-Aza, expression levels were upregulated in 6 out of 7 tumour cell line-derived CSCs. The LnCap CSCs showed moderate upregulation of the promoter region post treatment, but this was not statistically significant (Figure 2D). The *sFRP5* mRNA relative expression was significantly upregulated in 3 out of 7 tumour cell line-derived CSCs (Figure 2E).

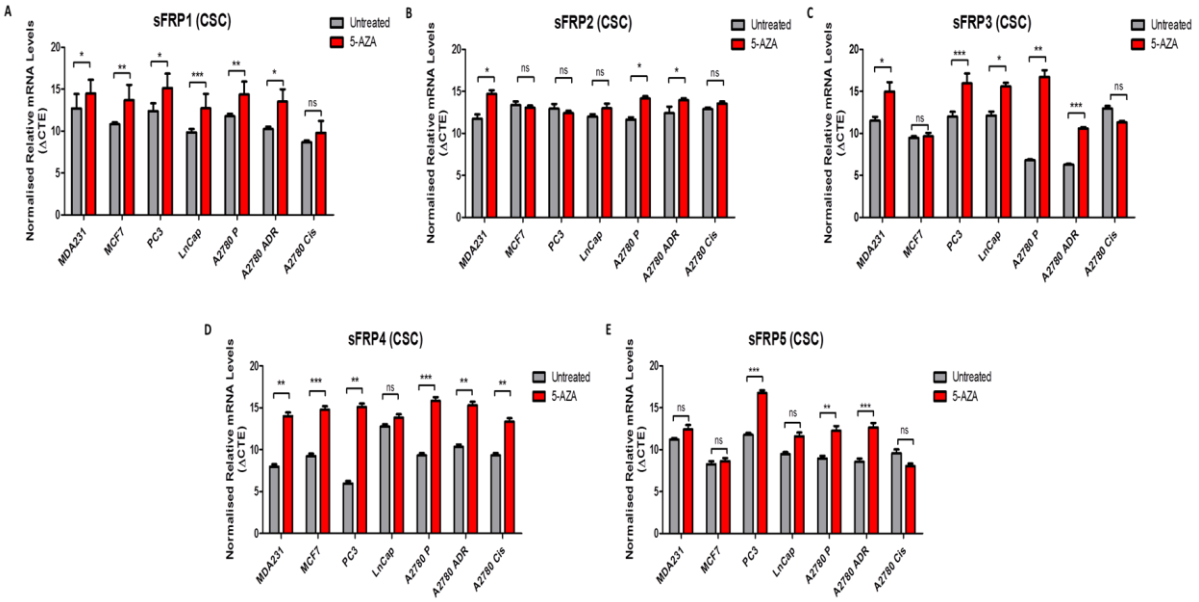


Figure 2: Effect of *DNMT1* inhibition on gene expression: Real-Time quantitative PCR results for sFRP1, 2, 3, 4, and 5 analysed in CSCs derived from breast (MDA231 and MCF7), prostate (PC3 and LnCap), and ovary (A2780 P, A2780 ADR, and A2780 CIS) tumour cell lines. Relative mRNA expression of *sFRPs (1-5)* following treatment of CSCs with 30 µM 5-Aza for 48 h treated CSCs to their untreated controls. Statistical analysis was performed using ANOVA for analysis variance with Bonferroni test for comparison showing significance as ***P < 0.001; **P < 0.01; *P < 0.05. Data are mean ± standard error of the mean from 3 independent experiments.

4.5.2 Methylation-specific PCR confirms *sFRP* promoter demethylation in CSCs.

To confirm the *sFRP* promoter region demethylation in CSCs of all originally methylated cell lines, the CSCs were treated with 5-Aza (30µM for 48 h) to induce demethylation of their genomic DNA (gDNA). Isolated gDNA was bisulfite treated to deaminate unmethylated cytosine to produce uracil in gDNA, allowing us to detect unmethylated cytosines and 5-methylcytosines using methylation specific PCR (MSP). We used the highly sensitive MSP to detect methylation patterns in *sFRPs'* (*1-5*) promoter regions to distinguish between methylated and unmethylated DNA from CSCs derived from all cells lines (Figure 3).

CSCs derived from all tumour cell lines exhibited a hypermethylated (M) promoter region in all *sFRPs* (*1-5*) in untreated cells, which confirmed the status of the methylated cell lines, except A2780 Cis in *sFRP1* (Figure 3A) and *sFRP3* (Figure 3C). Post 5-Aza treatment, most of the CSCs lacked the promoter methylation in analysed regions, indicating demethylation, except LnCap CSC in *sFRP4* (Figure 3D) and breast CSCs (MDA231/ MCF7 CSCs) in *sFRP5* (Figure 3E). The CSCs gaining an unmethylated promoter sequence after treatment consistently demonstrated upregulation of *sFRP* mRNA expression.

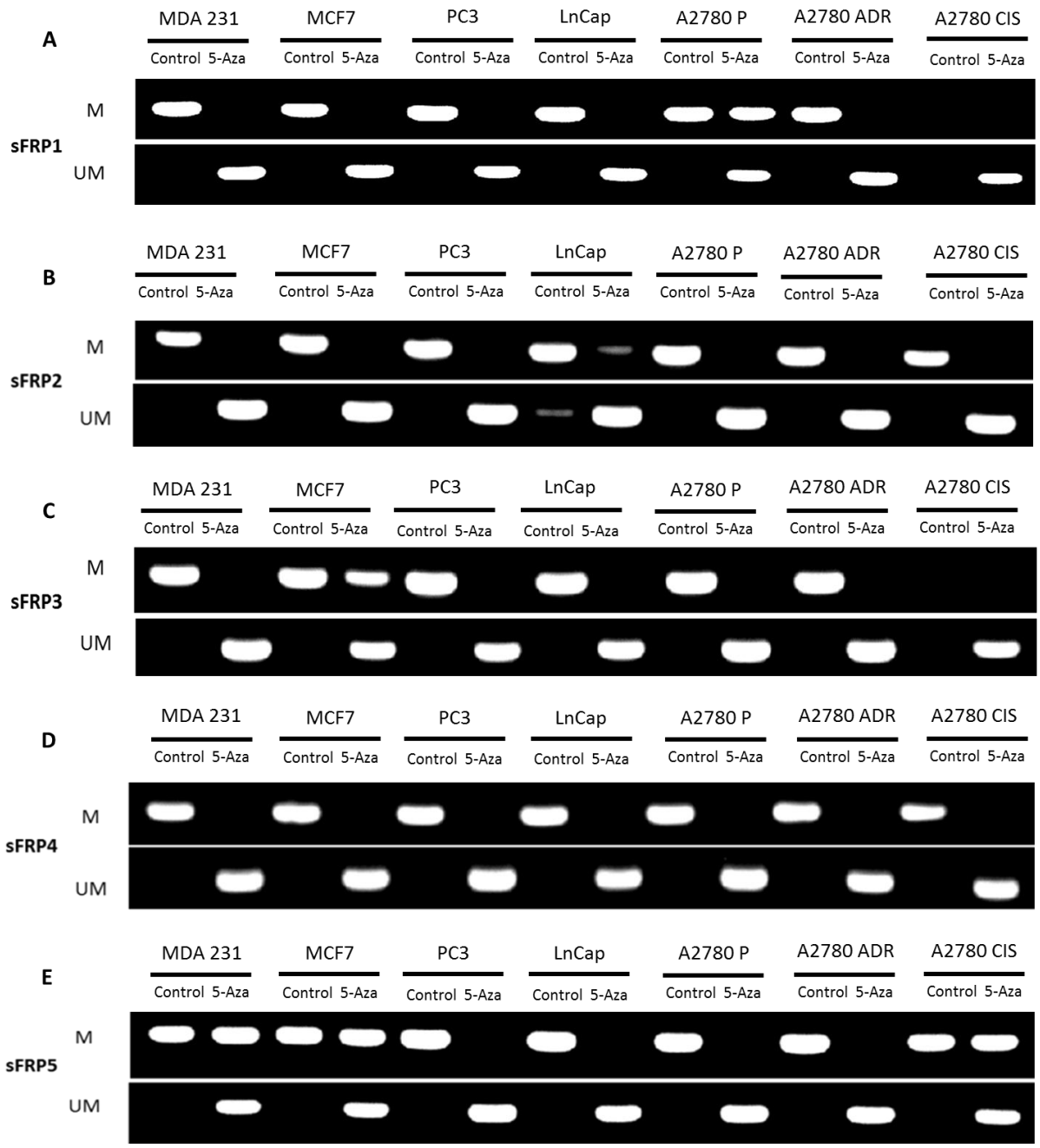
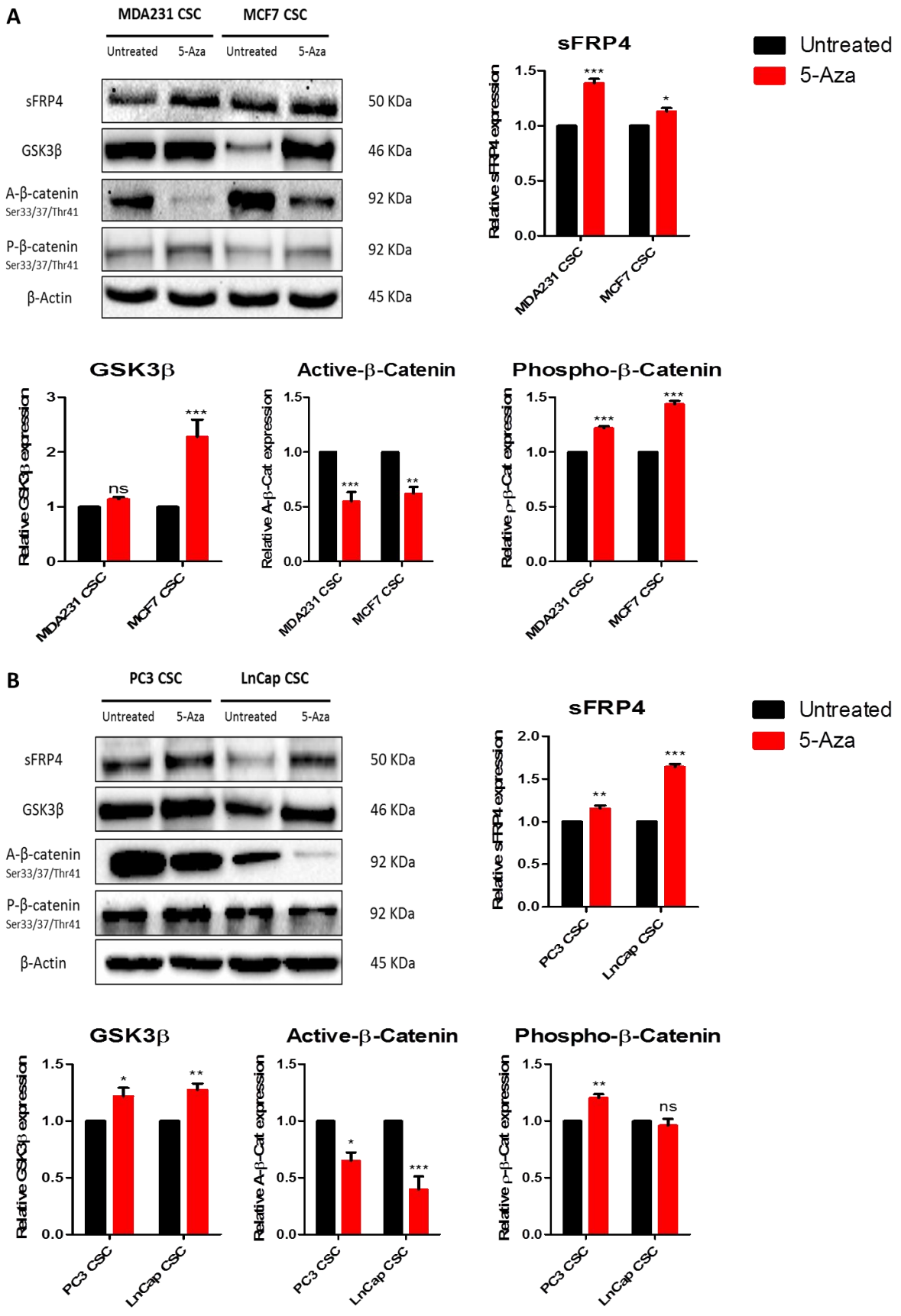


Figure 3: Effect of *DNMT1* inhibition on methylation: Methylation specific PCR (MSP) results for sFRP1, 2, 3, 4, and 5 analysed in CSCs derived from breast (MDA231 and MCF7), prostate (PC3 and LnCap), and ovary (A2780 P, A2780 ADR, and A2780 CIS) tumour cell lines. M: primer specific for methylated DNA. U: primer specific for unmethylated DNA. Semi-quantitative PCR images are representative of 3 independent experiments.

4.5.3 Demethylation regulates protein levels of Wnt signalling-associated molecules.

The loss of methylation in all *sFRP (1-5)* promoter regions and increased mRNA expression post 5-Aza treatment in all CSCs prompted us to investigate the effect of *DNMT1* inhibition on protein levels of the Wnt antagonist sFRP4 and other key downstream effectors of Wnt signalling such as GSK3β, active β-catenin, and phospho β-catenin. The Western blot results demonstrated the effects of 5-Aza against untreated control (Figure 4).

Following 5-Aza treatment, sFRP4 protein expression was significantly increased in all CSCs compared to the untreated CSCs, except in A2780 Cis CSCs (Figure 4C). The levels of GSK3β were increased in 4 out 7 CSC cell lines; MCF7 CSCs had more than 50% elevation in expression compared to untreated CSCs; A2780 CSCs showed elevation in GSK3β protein levels but this was not statistically significant (Figure 4C). Active β-catenin protein levels were significantly decreased in all CSCs compared to untreated CSCs, indicating the role of increased sFRP4 expression in deregulating Wnt activation. Phosphorylated β-catenin protein levels showed significant elevation in all CSCs, except LnCap CSCs (Figure 4B). The levels of downstream effectors in CSCs indicate that post 5-Aza treatment, increased sFRP4 levels enable GSK3β to phosphorylate β-catenin at ser33/37/Thr41, hence decreasing the level of active β-catenin.


A
MDA231 CSC
MCF7 CSC
Untreated
5-Aza
Untreated
5-Aza
sFRP4
GSK3β
A-β-catenin
Ser33/37/Thr41
P-β-catenin
Ser33/37/Thr41
β-Actin
50 KDa
46 KDa
92 KDa
92 KDa
45 KDa
sFRP4
Relative sFRP4 expression
MDA231 CSC
MCF7 CSC
Untreated
5-Aza
GSK3β
Relative GSK3β expression
ns
Active-β-Catenin
Relative A-β-Cat expression
Phospho-β-Catenin
Relative p-β-Cat expression
B
PC3 CSC
LnCap CSC
Untreated
5-Aza
Untreated
5-Aza
sFRP4
GSK3β
A-β-catenin
Ser33/37/Thr41
P-β-catenin
Ser33/37/Thr41
β-Actin
50 KDa
46 KDa
92 KDa
92 KDa
45 KDa
sFRP4
Relative sFRP4 expression
PC3 CSC
LnCap CSC
Untreated
5-Aza
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Relative GSK3β expression
Active-β-Catenin
Relative A-β-Cat expression
Phospho-β-Catenin
Relative p-β-Cat expression
ns

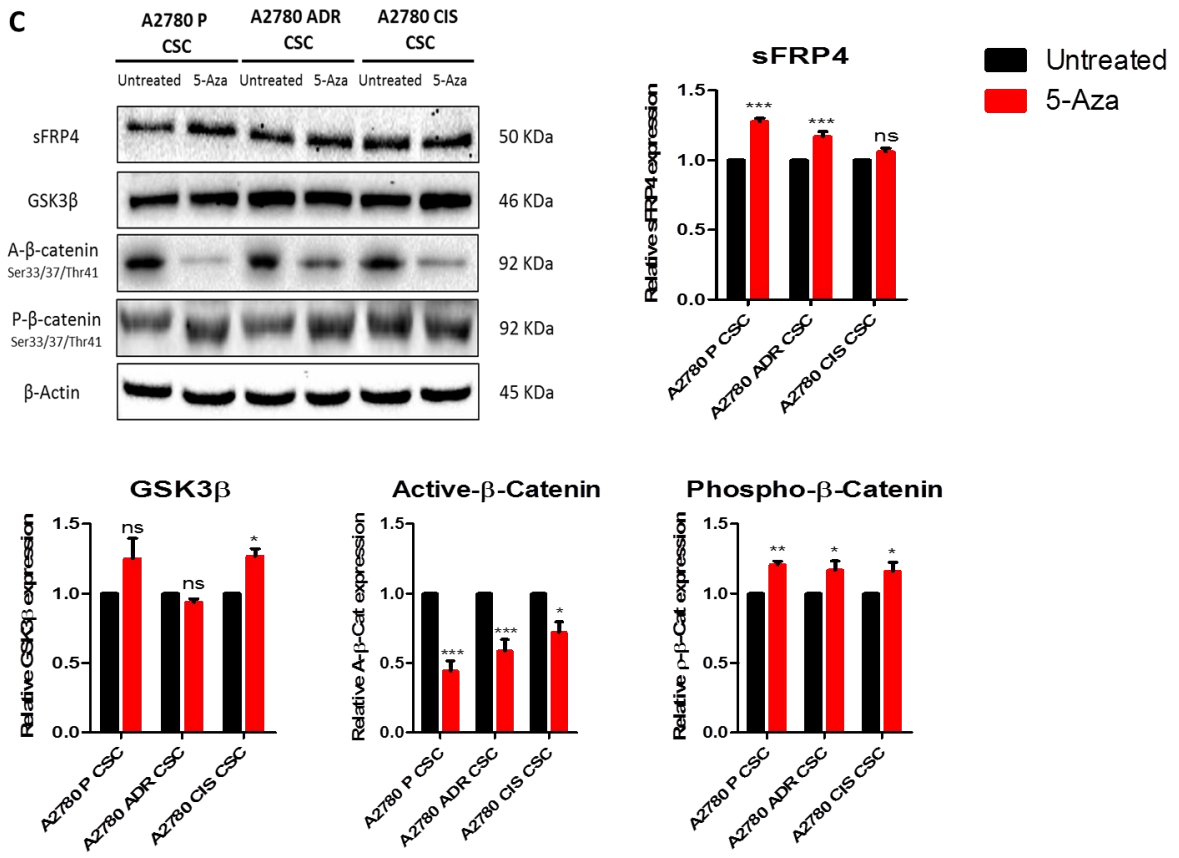


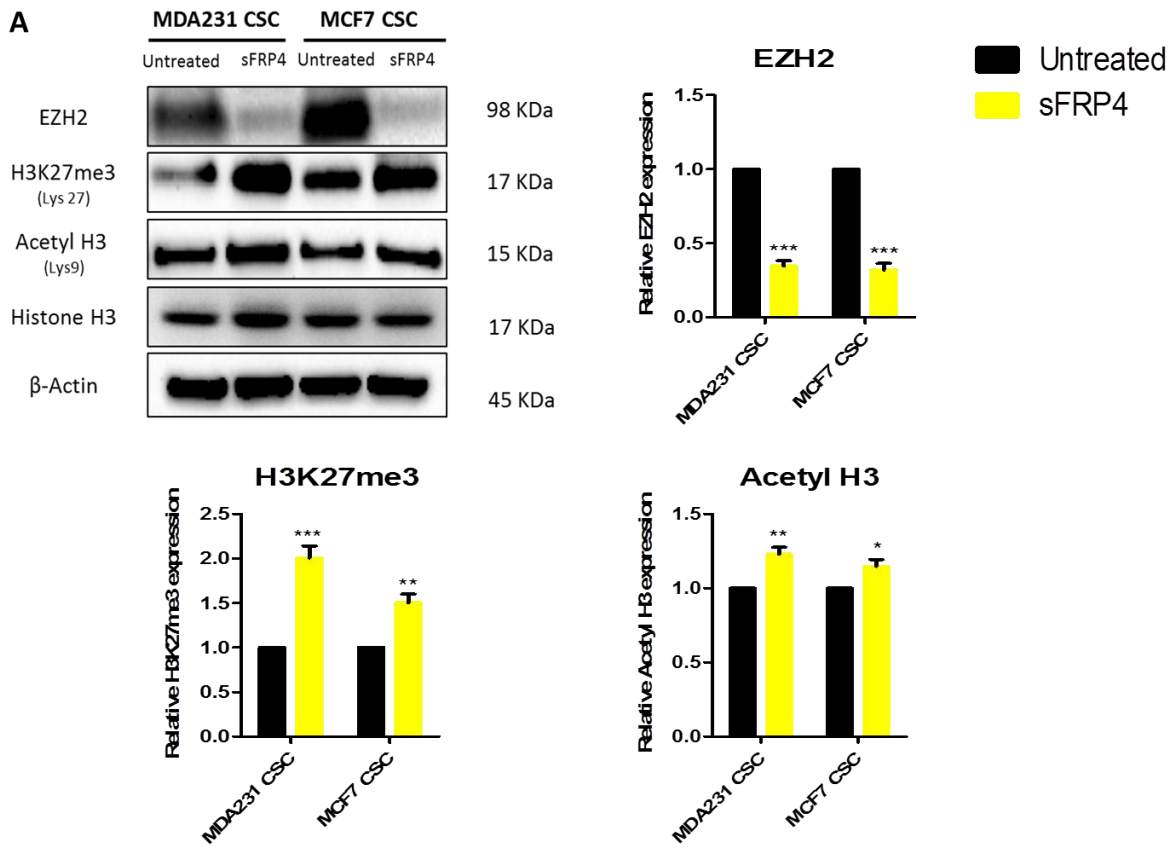
Figure 4: Effect of 5-Azacytidine on protein expression levels: Western blot results for sFRP4, glycogen synthase kinase 3 beta (GSK3β), active β-catenin, and phosphorylated β-catenin analysed in CSCs derived from A) breast (MDA231 and MCF7), B) prostate (PC3 and LnCap), and C) ovary (A2780 P, A2780 ADR, and A2780 CIS) tumour cell lines. Relative protein expression of Wnt associated molecules in CSCs when treated with 30 µM 5-Aza for 48 h. Statistical analysis was performed using ANOVA for analysis variance with Bonferroni test for comparison showing significance as ***P < 0.001; **P < 0.01; *P < 0.05. Blots and relative protein expressions are mean ± standard error of the mean from 3 independent experiments.

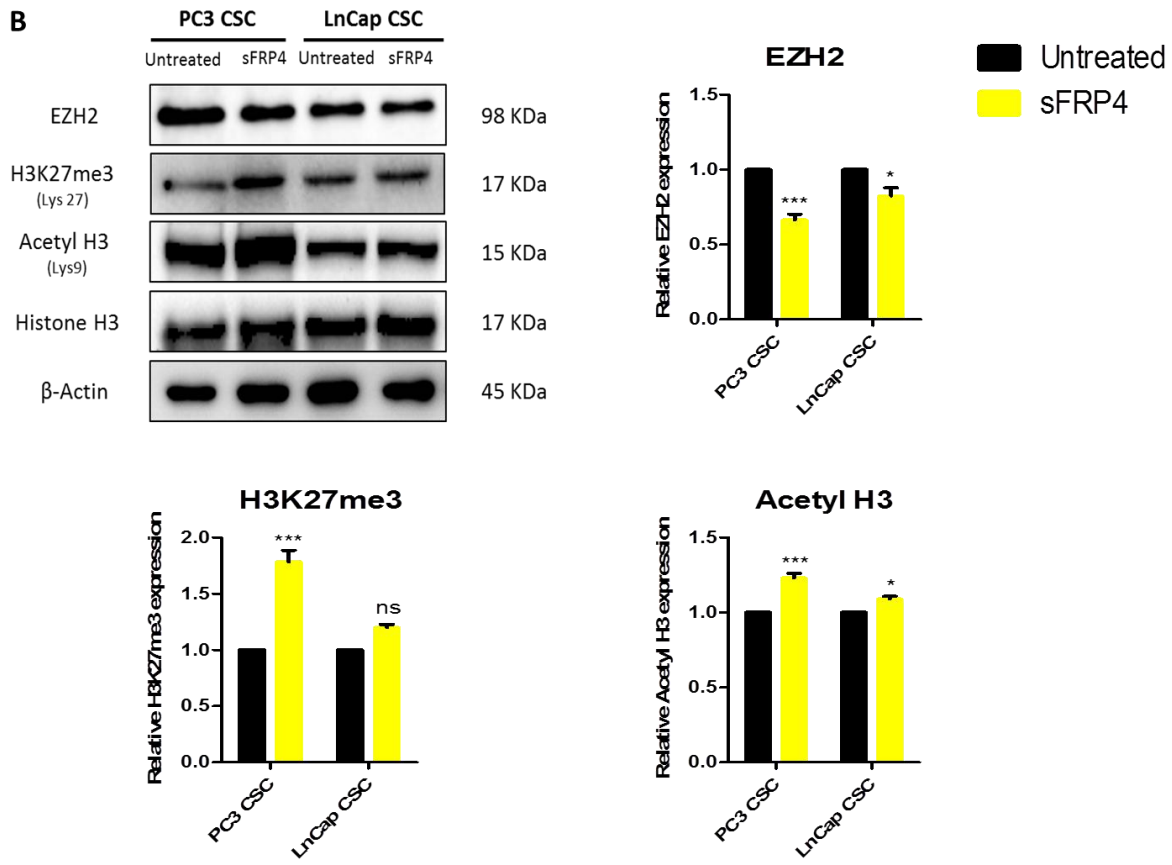
4.5.4 sFRP4 regulates histone modifications.

The increased levels of sFRP4 post 5-Aza treatment in CSCs prompted us to investigate the post-translational effect of sFRP4 on histone modification and its epigenetic co-factors such as acetylation and methylation of histone H3. We also investigated the effect of sFRP4 on enhancer zeste homolog 2 (EZH2), a histone methyltransferase. The Western blot results demonstrate the effects of sFRP4 against untreated control in CSCs (Figure 5).

Post sFRP4 treatment, EZH2 levels decreased significantly in all CSCs, except in A2780 ADR CSCs (Figure 5C). The sFRP4 treated CSCs exhibited increased levels of tri-methyl histone H3 (H3K27me3) in 4 out of 7 CSC cell lines, whereas LnCap CSCs (Figure 5B) and A2780 P CSCs (Figure 5C) demonstrated moderate increased levels, but these were not statistically significant. Acetyl histone H3 protein levels were increased in breast (MDA231/ MCF7) and prostate (PC3/ LnCap) CSCs.

The histone modification factors in CSCs post sFRP4 treatment indicate that decreasing EZH2 levels enables increased H3K27me3 levels, thereby inducing transcriptional repression.


A
MDA231 CSC
MCF7 CSC
Untreated
sFRP4
Untreated
sFRP4
EZH2
H3K27me3
(Lys 27)
Acetyl H3
(Lys9)
Histone H3
β-Actin
98 KDa
17 KDa
15 KDa
17 KDa
45 KDa
EZH2
Relative EZH2 expression
1.5
1.0
0.5
0.0
***
***
MDA231 CSC
MCF7 CSC
Untreated
sFRP4
H3K27me3
Relative H3K27me3 expression
2.5
2.0
1.5
1.0
0.5
0.0
***
**
MDA231 CSC
MCF7 CSC
Acetyl H3
Relative Acetyl H3 expression
1.5
1.0
0.5
0.0
**
*
MDA231 CSC
MCF7 CSC


B
PC3 CSC
LnCap CSC
Untreated
sFRP4
Untreated
sFRP4
EZH2
H3K27me3
(Lys 27)
Acetyl H3
(Lys9)
Histone H3
β-Actin
98 KDa
17 KDa
15 KDa
17 KDa
45 KDa
EZH2
Relative EZH2 expression
1.5
1.0
0.5
0.0
***
*
PC3 CSC
LnCap CSC
Untreated
sFRP4
H3K27me3
Relative H3K27me3 expression
2.0
1.5
1.0
0.5
0.0
***
ns
PC3 CSC
LnCap CSC
Acetyl H3
Relative Acetyl H3 expression
1.5
1.0
0.5
0.0
***
*
PC3 CSC
LnCap CSC

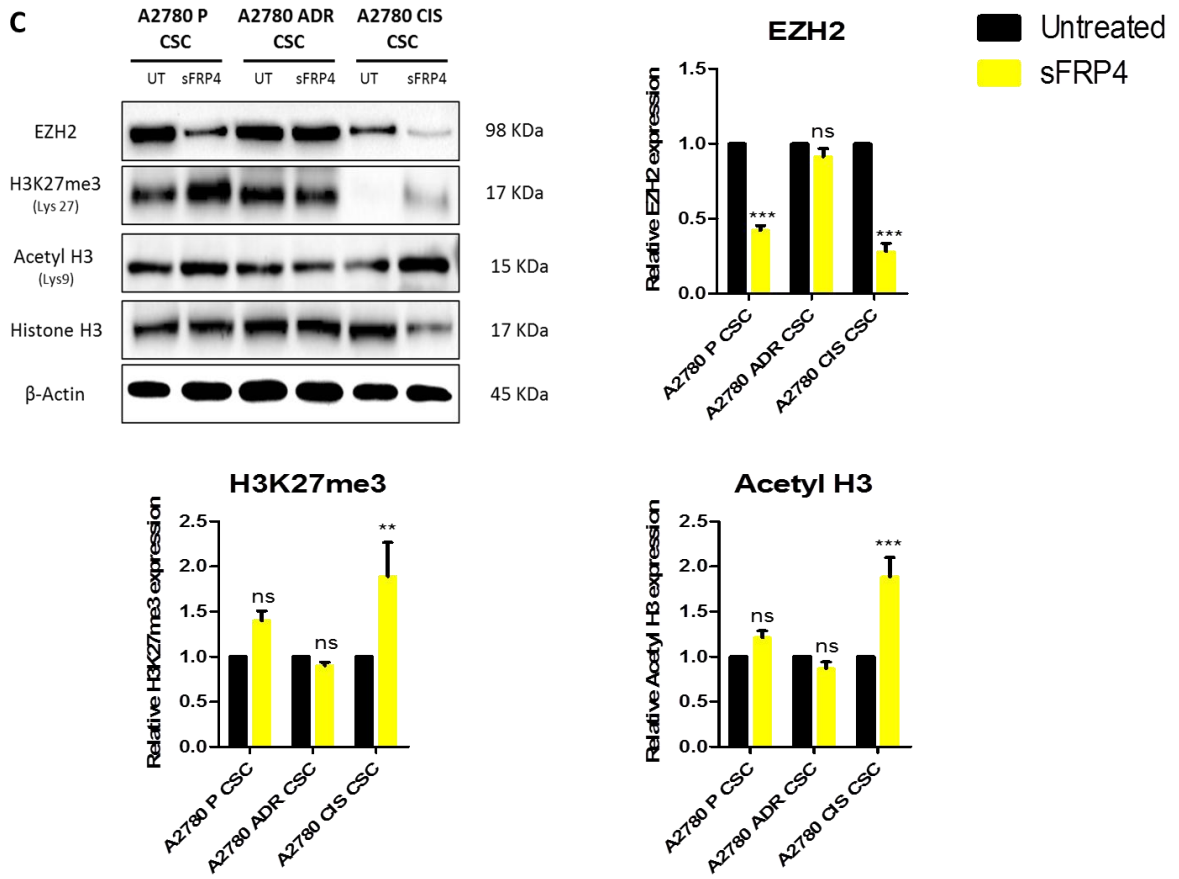


Figure 5: Effect of sFRP4 on histone modifications: Western blot results for enhancer of zeste homolog 2 (EZH2), Tri-Methyl-Histone H3 (Lys27) H3K27me3, Acetyl-Histone H3 (Lys9) H3K9ac, and total histone H3 in CSCs derived from A) breast (MDA231 and MCF7), B) prostate (PC3 and LnCap), and C) ovary (A2780 P, A2780 ADR, and A2780 CIS) tumour cell lines. Relative histone expression of 250 pg/ ml sFRP4 for 24 h treated CSCs to untreated CSCs. Statistical analysis was performed using ANOVA for analysis variance with Bonferroni test for comparison showing significance as ***P < 0.001; **P < 0.01; *P < 0.05. Blots and relative protein expressions are mean ± standard error of the mean from 3 independent experiments

4.6 Discussion

During tumor growth, many cancer cells differentiate to initiate tumorigenesis, though CSCs evade the cell cycle and remain in their resting phase (G0 Phase) (9, 31), preserving their self-renewal capacity acquired during transformation. Considering the importance of epigenetic alterations such as DNA methylation, histone modifications, and chromatin remodelling in stem cell maintenance, many DNA-methylating enzymes and histone modifiers are essential to maintaining the CSC functional state. The epigenetic silencing process such as DNA methylation of their gene promotor in CSCs has been previously reported (32). DNA methyltransferase 1 (*DNMT1*) regulates DNA methylation in human genes, which was demonstrated in a study involving a leukemic murine model, where wild type mice with *DNMT1* overexpression developed leukaemia, whereas *DNMT1* knockout did not (33-35). Loss of *DNMT1* expression can be associated with loss of genomic stability and viability of cellular function (36, 37), which implicates the hypomethylation of tumour suppressor genes. Epigenetic silencing of gene families has profound implications for understanding the mechanistic basis of carcinogenesis and CSC emergence. The cell-intrinsic mechanisms and functional characterizations responsible for maintaining cellular hierarchies within cancer are crucial in the epigenetic modulation of tumours and CSC progression.

The Wnt cell-cell signalling pathway tightly regulates self-renewal and differentiation (38). The downstream effectors of the Wnt signalling pathway can influence the crucial mediators of epigenetic factors that translate extracellular signalling into differential CSC phenotypes. The activation of canonical Wnt signalling in cancer is due to aberrant gene activation mediated through β-catenin (39). The sFRP genes (*sFRP1, 2, 3, 4*, and *5*), which are Wnt antagonists, are silenced due to promoter hypermethylation in human carcinogenesis (22, 23, 40, 41). *SFRP1* and *sFRP2* have been found to be epigenetically silenced in human brain cancer such as astrocytic gliomas (42), and *sFRP1* loss of expression has been attributed to promoter hypermethylation and allelic loss in papillary bladder cancer (43). Furthermore, *sFRP (1-5)* methylation was detected in liver cancer cell lines and primary hepatocellular carcinoma cells, indicating the methylation of these genes is an early event in liver carcinogenesis (44). In addition, aberrant CpG island methylation was found in *sFRP1, 2, 4*, and *5* in acute myeloid leukaemia cells (45). Methylation of *sFRP1, 2*, and *5* was frequently

detected in oral squamous cell carcinoma (OSCC), resulting in decrease of OSCC cell proliferation (46). In breast cancer cell lines, frequent methylation and silencing of *sFRP1, 2, 4*, and *5* has been observed, indicating the loss of sFRP functions contributes to activation of Wnt signalling in breast carcinogenesis (47). In glioblastoma multiforme cell lines, all *sFRP* isoforms were epigenetically silenced and were demethylated using the DNMT inhibitor 5-Azacytidine (22).

In order to gain further insights into epigenetic alterations in CSCs, we investigated the methylation status of all sFRP family genes in CSCs derived from various tumour cell lines. Our results demonstrated DNA demethylation using the *DNMT* inhibitor, 5-Aza in all CSCs; furthermore demonstrating that sFRP4 demethylation in CSCs would decrease the protein levels of Wnt signalling-associated molecules. We also investigated the activation of sFRP4 in CSCs to study histone modifications, demonstrating a correlation between promoter hypermethylation and post-translational modifications in CSCs derived from all the cell lines. The CSCs were identified from breast (MDA231 and MCF7), prostate (PC3 and LnCap), and ovary (A2780P, A2780 ADR, and A2780 CIS) tumor cell lines based on their ability to form spheroids in serum free conditions, elevated expression of CSC surface markers, high expression of ABC drug transporters (ABCG2), cell survival protein (Cyclin D1), oncogenes (c-Myc), and the ability to escape cell death/apoptosis (Bcl-xl) (7). Hypermethylation of *sFRP1, 2, 3, 4*, and *5* was detected in all CSCs examined before demethylation of their gDNA through incubation with 30µM 5-Azacytidine for 48 h.

The *sFRP1* promotor regions are hypermethylated in renal cell carcinoma, as evidenced by downregulation of mRNA levels compared to normal kidney tissues. The complete loss of sFRP1 at the protein level substantiated its methylation mediated silencing (48). We observed a similar outcome post 5-Aza treatment; there is an increase in mRNA levels of *sFRP1* promoter regions, indicating CSC DNA demethylation in 6 out of 7 cell lines. In addition, we observed *sFRP* mRNA abundance before and after 5-Aza treatment of CSCs, revealing significant upregulation of *sFRP2* (3 out of 7 cell lines), *sFRP3* (5 out of 7 cell lines), *sFRP4* (6 out of 7 cell lines), and *sFRP5* (3 out of 7 cell lines). The mRNA levels post-treatment indicate that sFRPs are transcriptionally silenced by epigenetic processes in CSCs. Moreover, we showed loss of methylation within sFRPs' promotor regions after treatment with 5-Aza, and CSCs gaining an unmethylated promoter sequence after treatment consistently showed upregulation of *sFRP* mRNA expression. Therefore, the findings suggest

that epigenetic processes mediated through methylation of 5`-CpG-islands in the promotor regions of sFRPs are involved in epigenetic alterations within CSCs derived from the tumour cell lines examined.

In human mesothelioma cell lines, sFRP4 protein levels were decreased for all the hypermethylated promoter regions, indicating the correlation of epigenetic modulations to post-translational modification in the Wnt signalling pathway (49, 50). In another study, treating myeloid leukaemia cells with 5-Aza induced the expression of previously methylated *sFRP1, 2, 4*, and *5*, and resulted in inactivation of the Wnt pathway by downregulating important Wnt genes such as *cyclin D1, TCF1*, and *LEF1* (51). Our Western blot analyses demonstrated increased sFRP4 expression, GSK3β expression, and phosphorylated β-catenin expression after demethylating treatment, which confirms our hypothesis that methylation, appears to functionally silence sFRP4 gene expression in CSCs. To further validate the relation between demethylation and inactivation of Wnt signalling, we analysed the important Wnt pathway-associated molecules, demonstrating a decrease in protein levels of active (unphosphorylated) β-catenin.

Post-translational modifications (PTM) of N-terminal tails of histones, which includes methylation, acetylation, ubiquitination, and phosphorylation, can modulate transcription factors and transcriptional activity of genes (52). The regulation of genetic materials in cancer is controlled by histone modifications and is suspected to be involved in tumour progression. In our study, we further investigated the role of sFRP4 in histone H3 modifications such as methylation and acetylation.

The polycomb repressive complex 2 (PRC2) and DNA methylation in CSCs can supress their differentiation capacity (53). PRC2 consists of enhancer zeste homolog 2 (EZH2), which acts as a catalyst for tri-methylation of histone 3 lysine 27 (53). In CSCs, the chromatins recruit DNMTs, leading to *ex novo* methylation and gene silencing, and leading to silencing of tumour suppressor genes during tumour progression; however, H3K27 can also silence tumour genes without DNMT recruitment (54). The polycomb gene complex (PcG) target genes are more likely to be hypermethylated in colon and embryonic carcinoma cell lines (55-57), indicating that EZH2 recruits DNMT to promoter regions of PcG target regions and silences tumour suppressor genes (58). These studies establish the crosstalk between DNA methylation and histone modifications, which result in a repressive effect. Our findings substantiate the crosstalk between DNMT inhibition and histone modifications, exemplified

by the decreased levels of EZH2 post sFRP4 treatment and upregulation of *sFRP4* post DNMT1 inhibition. The sFRP4 treatment led to increased methylation of the H3K27me3 target and suppression of EZH2, highlighting the important role of EZH2 in self-renewal of CSCs. EZH2 plays an important role in CSCs, including repression of tumour suppressor genes (59-62), and β-catenin stabilization (63). As a consequence of the cellular changes induced by transcription and epigenetic alterations, the CSCs become dependent on EZH2 to preserve their self-renewal capacity (61).

We also investigated the effect of sFRP4 activation on Histone H3 acetylation, as it is an important modification related to DNA repair, cell cycle regulation, and transcription activation (64). Addition of an acetyl group in transcription causes DNA denaturation, which is a hallmark for cancer cell therapy. Deficiency of Histone H3 acetylation has been detected in tumour progression of prostate cancer patients (65), and hyper-acetylation of H3K56 has been reported in hepatocellular carcinoma (66). The naturally occurring compound curcumin induced histone H3 and H4 acetylation, leading to apoptotic cell death in brain cancer (67). In our study, we observed that pro-apoptotic sFRP4 led to acetylation in all CSCs, affecting their self-renewal capacity and tumour progression.

4.7 Conclusion

Considering the epigenetic diversity seen in cancers, modulations among CSCs are still under scrutiny. The prospect of epigenetic alterations in CSCs is important in cancer research as it has led to the approval of clinical drugs inhibiting epigenetic modifiers, such as histone deacetylases (HDAC) and methyltransferases (HMT) (68-75). In summary, our data suggest that epigenetic silencing of *sFRPs* in CSCs derived from breast, prostate, and ovary tumour cell lines is most likely due to hypermethylation of their promoter regions. Activation of *sFRP4* by inhibiting DNA methyltransferase indicated the influence on downstream effectors of Wnt-associated molecules. Taking into account the pro-apoptotic and chemo-sensitization capability of sFRP4, sFRP4-led epigenetic modulations and histone modifications in CSCs might serve as a potential therapeutic approach towards CSCs.

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Chapter 5

Deshmukh, A., Arfuso, F., Newsholme, P., & Dharmarajan, A. (2017). Regulation of cancer stem cell metabolism by secreted frizzled related protein-4 (sFRP4).

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5 Regulation of cancer stem cell metabolism by secreted frizzled-related protein 4 (sFRP4)

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Keywords

Cancer stem cells; Metabolism; Secreted fizzled-related protein; Glycolysis; Glucose; Apoptosis.

Abstract

Tumours contain a small number of treatment-resistant cancer stem cells (CSCs), and it is through these tumour regrowth originates at secondary sites, thus rendering CSCs an attractive target for novel treatment. Cancer cells adapt cellular metabolism for aggressive proliferation. Tumour cells use less efficient glycolysis for the production of ATP and increasing tumour mass (Warburg effect), instead of oxidative phosphorylation (OXPHOS). CSCs show distinct metabolic shift and, depending on the cancer type, can be highly glycolytic or OXPHOS dependent. Since Wnt signalling promotes glycolysis and tumour growth, we investigated the effect of the Wnt antagonist secreted frizzled-related protein 4 (sFRP4) on CSC metabolism. We demonstrate that sFRP4 has a prominent role in basal glucose uptake in CSCs derived from breast, prostate, and ovary tumour cell lines. We show that sFRP4 treatment on CSCs isolated with variable glucose content induces metabolic reprogramming by relocating metabolic flux to glycolysis or OXPHOS. Altogether, sFRP4 treatment compromises cell proliferation and critically affects cell survival mechanisms such as viability, glucose transporters, pyruvate conversion, mammalian target of rapamycin, and induces CSC apoptosis under conditions of variable glucose content. This provides the feasibility of using sFRP4 to inhibit CSC survival in order to induce metabolic reprogramming *in vivo.*

5.1 Introduction

Accumulating evidence suggest that tumours of various tissue origins, including breast, prostate, and ovary contain a small sub-population of cells with stemness capacity, often referred to as cancer stem cells (CSCs) or tumour initiating cells (TICs) [1-4]. In addition to the CSCs' self-renewal and migratory capacity, they also possess the ability to efflux toxic compounds and chemotherapeutic agents due to their high expression of ATP-dependent efflux pump ABCG2, high DNA repair system, and activation of survival cascades [5-7].

Current research has established certain key components and signalling pathways that affect the stemness and differentiation of CSCs [8-11], although the effect of nutrients and metabolites on CSCs remains elusive. A recent study suggests that CSCs have particular metabolic properties enabling their identification from the bulk tumour cells based up their biochemical profile [12]. Another study demonstrated that brain CSCs exhibit low mitochondrial respiratory activity and prefer a hypoxic environment to maintain their stemness [11]. Glioma stem cells (GSCs) were glycolysis driven and were intrinsically sensitive to the use of a glycolytic inhibitor [13]. However, cancer cells prefer glycolysis for their ATP production, and CSCs appear to have higher glycolytic activity. The Warburg hypothesis is consistent with the CSCs' dependency on glycolysis and switching on oxidative phosphorylation to facilitate cytosolic glycolysis [14, 15]. Based on these observations that glucose is an essential nutrient for CSCs, we reasoned that glucose might have a significant effect on the CSC subpopulation in bulk tumour cells. Furthermore, this enabled us to evaluate CSC survival under conditions of variable glucose content, and we also investigated the role of Wnt antagonism to regulating CSC survival in these conditions.

Wnt signalling plays an important role in tissue development and maintenance of normal tissues, though aberrant Wnt signalling activation is implicated in many cancers [16]. Wnt signalling in the "ON" state leads to active β-catenin accumulation in the nucleus and its interaction with LEF/ TCF (lymphoid enhancer factor/ T-cell factor) transcription factors leads to activation of Wnt target genes that are important for cancer cell survival [17, 18]. Aberrant Wnt signalling has been implicated in tumorigenesis, as its downstream targets are involved in cell survival, differentiation, and proliferation; therefore, inhibition of the Wnt pathway is a potential strategy for halting tumour progression. Secreted frizzled-related

protein 4 (sFRP4) is a Wnt antagonist inhibiting canonical Wnt signalling by binding to Wnt ligands and frizzled receptors [19]. Our previous studies have identified sFRP4's ability to inhibit multiple functional outputs of oncogenic Wnt signalling in CSCs, including a decrease in viability and epithelial-mesenchymal transition (EMT) induction, inhibiting angiogenesis, inducing apoptosis, and modulating cell survival [8, 20]. Here, we propose a novel function of sFRP4 in the regulation of CSC metabolism.

In this study, we used cell lines from breast (MDA231 and MCF7) and prostate (PC3 and LnCap) tumours to isolate CSCs as an *in-vitro* model. We investigated the effect of sFRP4 on CSCs isolated in culture medium without, low, and high glucose content. The addition of glucose to the culture medium induced a significant increase in CSC viability, which was decreased post sFRP4 treatment under the same conditions. We also demonstrated that the CSC metabolic profile changes with increasing glucose content in culture medium, and Wnt signalling plays a key role in mediating glucose induced CSC survival. Finally, we investigated the potential therapeutic effect of sFRP4 on CSC viability, glucose uptake, glutamine uptake, glutamate secretion, NAD+/ NADH ratio, and metabolically relevant proteins, and showed that sFRP4 compromised CSC viability and impairing CSC survival by initiating apoptosis regardless of glucose content.

5.2 Materials and Methods

5.2.1 Cell Culture

5.2.1.1 Monolayer cell culture

Cell culture plates for adherent cells were purchased from Nunc™ (ThermoFisher Scientific). The human breast tumour cell lines MDA-MB 231 (ER-) and MCF-7 (ER+) and human prostate tumour cell lines PC-3 (AR-/PSA-) and LnCap (AR+) were purchased from American Type Culture Collection (ATCC, USA). The cells were cultured in RPMI-1640 medium (Gibco #11875-093) supplemented with 10% foetal bovine serum (Bovogen #SFBS) and 100 U/ml PenStrep (Life Technologies #15070063). All cells were maintained at 37°C in a humid incubator with 5% CO_2.

5.2.1.2 Cancer stem cell isolation

For CSC isolation, culture plates with an ultra-low-attachment surface were purchased from Corning Life Sciences. CSCs were cultured in serum-free medium (SFM) containing DMEM-No Glucose (Gibco, US #11966025), DMEM-Low Glucose, 5.5 mM (HyClone, USA #SH30021.01), and DMEM-High Glucose, 25 mM (HyClone, USA #SH30081.02) supplemented with the growth factors bFGF (20 ng/ml) (ProSpec Bio #cyt-085), EGF (20 ng/ml) (ProSpec Bio #cyt-217), and 1× B27 (Gibco #17504044), and 100 U/ml PenStrep (Life Technologies #15070063). CSC-enriched populations of cells were obtained by plating a single cell suspension of breast and prostate tumour cells at 10000 cells/cm² in SFM on Low-adherent six-well plates (Corning #3471). CSCs were isolated in SFM; the spheroids are formed at the 3rd day of plating tumour cells. To analyse the effects of sFRP4, cells were cultured in medium supplemented with sFRP4.

5.2.2 CSC Treatment

The CSCs were treated in this study with purified sFRP4 (R&D Systems #1827-SF-025). CSC sensitization with sFRP4 was performed by adding sFRP4 to the cell culture at 250 pg/ml [8] for 24 h at 37°C in a 5% CO_2 incubator.

5.2.3 Viability Assay

A cell counting viability kit (CCK8, Sigma- Aldrich #96992) was used for the quantitation of viable cells. Monolayer cells were plated with culture medium varying in glucose content at a density of 10000 cells/cm² in a low-adherent flat-bottomed 96-well plate (Corning #3474) for 3 days in non-adherent SFM conditions. Wells with treatment-free medium were used as a negative control. CSCs were treated with sFRP4 for 24 h, then 10 µl of CCK8 solution was added to each well and incubated at 37°C in a 5% CO_2 incubator for 1 h. Plates were read at 450 nm using an EnSpire Multilabel Plate Reader (Perkin-Elmer).

5.2.4 Cell Surface Markers

To assist in determining their identity, cell surface markers were examined in CSCs by flow cytometry (BD FACSCANTO II) using CellQuest data acquisition and analysis software. APC-CD44 (1:100) (BioLegend #338805), PE Cy7-CD24 (1:10) (BioLegend #311119), and PE-CD133

(1:100) (BioLegend #372803). Cells incubated with conjugated irrelevant IgGs were used as negative controls. Tumour specific CSC markers used were: breast CSCs ($CD44^+$/ $CD24^{-/low}$) [4, 21] and prostate CSCs ($CD133^+$/ $CD44^+$) [22]. CSCs were characterized by flow cytometry (BD FACSCANTO II), as previously published [8]. These data are not shown since the surface markers were used only to characterize CSCs and we did not see an effect of sFRP4 on CSC cell surface marker expression.

5.2.5 The Cancer Genome Atlas Dataset

To analyse the relationship between *sFRP4* and *AMPKB1* (AMP Kinase), *mTOR* (Mammalian target of rapamycin), *GLUT1* (glucose transporter), *SLC1A5* (Glutamine transporter), *BAD* (Bcl-2 associated death promotor), and *PDHA1* (Pyruvate dehydrogenase) in breast and prostate cancers, we obtained data from The Cancer Genome Atlas (TCGA project), Nature 2011 by using www.cbioportal.org [23, 24]. On the home page of the website, select 'download data', then select for breast "Breast Invasive Carcinoma (TCGA, Nature 2012)",and for prostate "Prostate Adenocarcinoma (TCGA, Cell 2015)", click "mRNA expression Z-score (all genes)" from select genomic profiles and enter gene set for e.g.: "sFRP4 AMPKB1", select "Transpose data matrix" and click submit. The sFRP4 and AMPKB1 (encoding AMPK) mRNA Z-scores for 825 cases (Breast) and 333 cases (Prostate) will appear. The same process was followed for all the genes examined. The correlation between these Z-scores of two genes was then analyzed by Spearman correlation and Pearson correlation, and plotted using GraphPad Prism V5.0 (GraphPad software, La Jolla, USA).

5.2.6 Glucose uptake in CSCs

The bioluminescent glucose uptake assay was applied to CSCs in 96-well low adherent white luminescent plates. Before beginning the assay, the culture medium was removed and the CSCs were washed with 100 µl of phosphate-buffered saline (PBS). To initiate glucose uptake, 50 µl of 2-Deoxy-D-Glucose (2DG) (1mM) in PBS was added to cells. The uptake reaction was stopped and samples were processed as described in the standard protocol of the Glucose Uptake Glo Assay kit (Promega, USA #J1342). Because glucose uptake is time dependent, the optimal assay time was determined by stopping the reaction at the 90 mins point. This was the time frame that was chosen for standard glucose uptake conditions in

CSCs. The luminescent signal produced by this assay is proportional to the rate of glucose uptake, but the precise rate of glucose uptake can be calculated by taking into account the number of cells (10000 cells/ well), time of uptake (90 mins), and the amount of 2-Deoxy-D-Glucose-6-phosphate (2DG6P) produced (µM) as measured using the standard protocol. The rate of glucose uptake was measured as fmol/min/cell [25]. Luminescence was read with 0.3-1 second integration on a luminometer (EnSpire Multilabel Plate Reader, Perkin-Elmer).

5.2.7 Detection of extracellular metabolites in CSC medium

For measuring changes in glutamine and glutamate in the CSC medium, CSCs were isolated from MDA231, MCF-7, PC-3, and LnCap cells and plated in SFM conditions in low adherent 96-well plates at a density of 10000 cells/ cm^2. CSCs were grown in 100 µl DMEM medium supplemented with variable glucose concentrations, 4 mM glutamine, and growth factors (see CSC isolation). The cells were incubated in a tissue culture incubator (37°C, 5% CO_2), and were treated with sFRP4 (250 pg/ml) on the 3rd day for 24 h. At the indicated time (i.e. 24 h treatment), 2 µl of culture medium was removed and transferred to a separate 96 well-plate containing 98 µl PBS/well. For metabolite analysis, 4.5 µl of thawed sample were transferred to respective 96 well white luminescent plates for glutamine and glutamate detection. Samples were then assayed as described in Glutamine/Glutamate-Glo Assay (Promega, USA #J8021) standard protocols [26]. The luminescence was read on a luminometer (EnSpire Multilabel Plate Reader, Perkin-Elmer).

5.2.8 Detection of qualitative NAD^+/NADH in CSCs

The NAD^+/NADH ratio was quantified by a luciferase assay provided in the NAD^+/NADH Glo Assay kit (Promega, USA #G9071). CSCs were isolated from MDA231, MCF-7, PC-3, and LnCap cells plated in SFM conditions in low adherent 96-well plates at a density of 10000 cells/ $cm^{2.}$ The cells were incubated in a tissue culture incubator (37°C, 5% CO_2), and were treated with sFRP4 (250 pg/ml) on the 3rd day for 24 hr. Briefly, after appropriate treatment over the desired time, the medium was removed and cells were supplemented with 50 µl of PBS and 50 µl of 0.2 N NaOH solution with 1% DTAB to obtain a cell lysate. To measure NAD^+, a 50 µl aliquot of cell lysate was treated with 0.4 N HCl and heat quenched at 60°C for 15 mins. The solution was neutralized with the Trizma buffer. NADH samples were heat

quenched following the addition of NaOH with 1% DTAB and the solution was neutralized with HCl-Trizma. An equal volume of NAD/NADH-Glo Detection Reagent was added to each well with cell lysate, incubated at room temperature for 60 min, and the luminescence was read on a luminometer (EnSpire Multilabel Plate Reader, Perkin-Elmer).

5.2.9 Western Blotting

CSCs were washed twice with PBS and then lysed in RIPA lysis buffer (Sigma #R0278) (150 mM NaCl, 1.0% IGEPAL® CA-630, 0.5% sodium deoxycholate, 0.1% SDS, 50 mM Tris pH 8.0, Proteinase Inhibitor 1x). Post sonication, cell lysates were centrifuged at 14000g for 10 min at 4 °C, and the supernatants were used for Western blotting. The lysates were resolved by sodium dodecyl sulphate-polyacrylamide gel electrophoresis, transferred onto nitrocellulose membranes, and then stained with 0.1% Ponceau S solution (Sigma #P3504) to ensure equal loading of the samples. After being blocked with 5% non-fat milk for 60 min, the membranes were incubated with primary antibodies mTOR (7C10) (1:1000, Cell Signaling #2983); AMPKα (1:1000, Cell Signaling #2532); Acetyl-CoA Carboxylase (C83B10) (1:1000, Cell Signaling #3676); Fatty Acid Synthase (C20G5) (1:1000, Cell Signaling #3180); Pyruvate Dehydrogenase (C54G1) (1:1000, Cell Signaling #3205); GLUT4 (1:2500, Abcam #ab65267); BAD (1:500, SantaCruz #sc-8044); and β-Actin (13E5) (1:1000, Cell Signaling #4970) overnight at 4°C, and the bound antibodies were visualized with horseradish peroxidase-conjugated secondary antibodies using the ECL Western Blotting Substrate (Amersham, GE #RPN2106) on a Chemi-Doc (Bio-Rad) imaging analyser.

5.3 Results

5.3.1 The sphere forming capacity of CSCs is reduced by sFRP4 irrespective of glucose content.

To investigate the potential phenotypic changes that glucose dependency might induce in CSCs, we evaluated CSC morphology in different culture media containing various glucose levels. CSCs were treated with sFRP4 (250 pg/ml) for 24 hr. The untreated spheroids remained intact, whereas the sFPR4 treated cells showed disruption of spheroids (Figure 1) in No Glucose/ Low Glucose/ High Glucose culture medium. High glucose content in the culture medium provided favourable conditions for CSCs to form spheroids and to survive in a quiescent state; however, sFRP4 segregated the spheroids in MCF-7, PC-3, and LnCap CSCs (Figure 1). Without glucose, CSCs spheroids are more stressed and susceptible to sFRP4`s segregation capacity. CSCs isolated in low glucose culture medium showed a similar effect as in no glucose, indicating that glucose is critical for CSC survival. However, sFRP4 segregated the spheroids in all glucose conditions, indicating that sFRP4's effects are not influenced by glucose conditions.

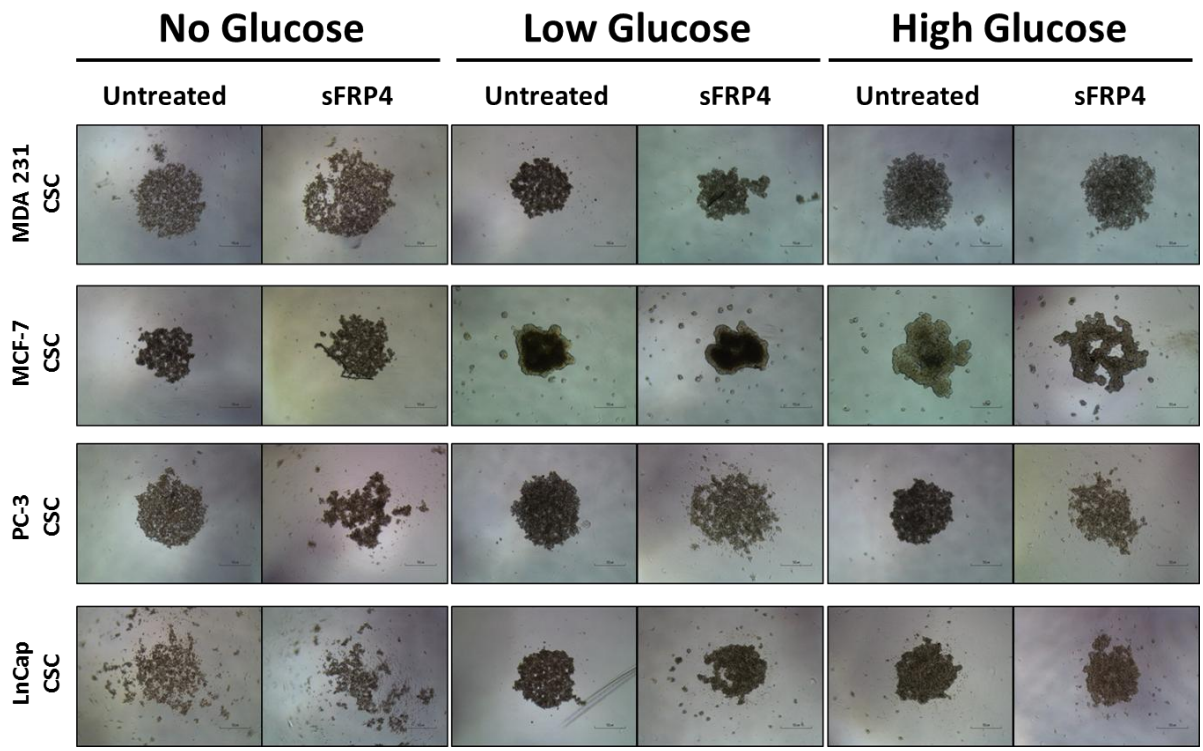


Figure 1: The effect of sFRP4 on CSC morphology: CSCs were isolated from breast and prostate tumour cell lines with increasing glucose concentrations and treated with sFRP4 (250pg). The sFRP4 treatment results in disruption of the CSC sphere. (Scale bar: 100 µm). Images are representative of all the experiments.

5.3.2 CSC viability reduced by sFRP4 in Low and High Glucose conditions

Using a CCK-8 viability assay, it was observed that the viability of CSCs increased with increasing glucose content in the culture medium (Figure 2). This indicates the requirement of glucose for CSC metabolism and survival. However treatment with sFRP4 significantly inhibited the viability of CSCs in low and high glucose conditions compared to untreated CSCs. In low glucose conditions, sFRP4 critically affected the viability in MDA231 (Figure 2A), MCF-7 (Figure 2B), and LnCap CSCs (Figure 2D), whereas a minimal effect was observed in no-glucose conditions for all CSCs.

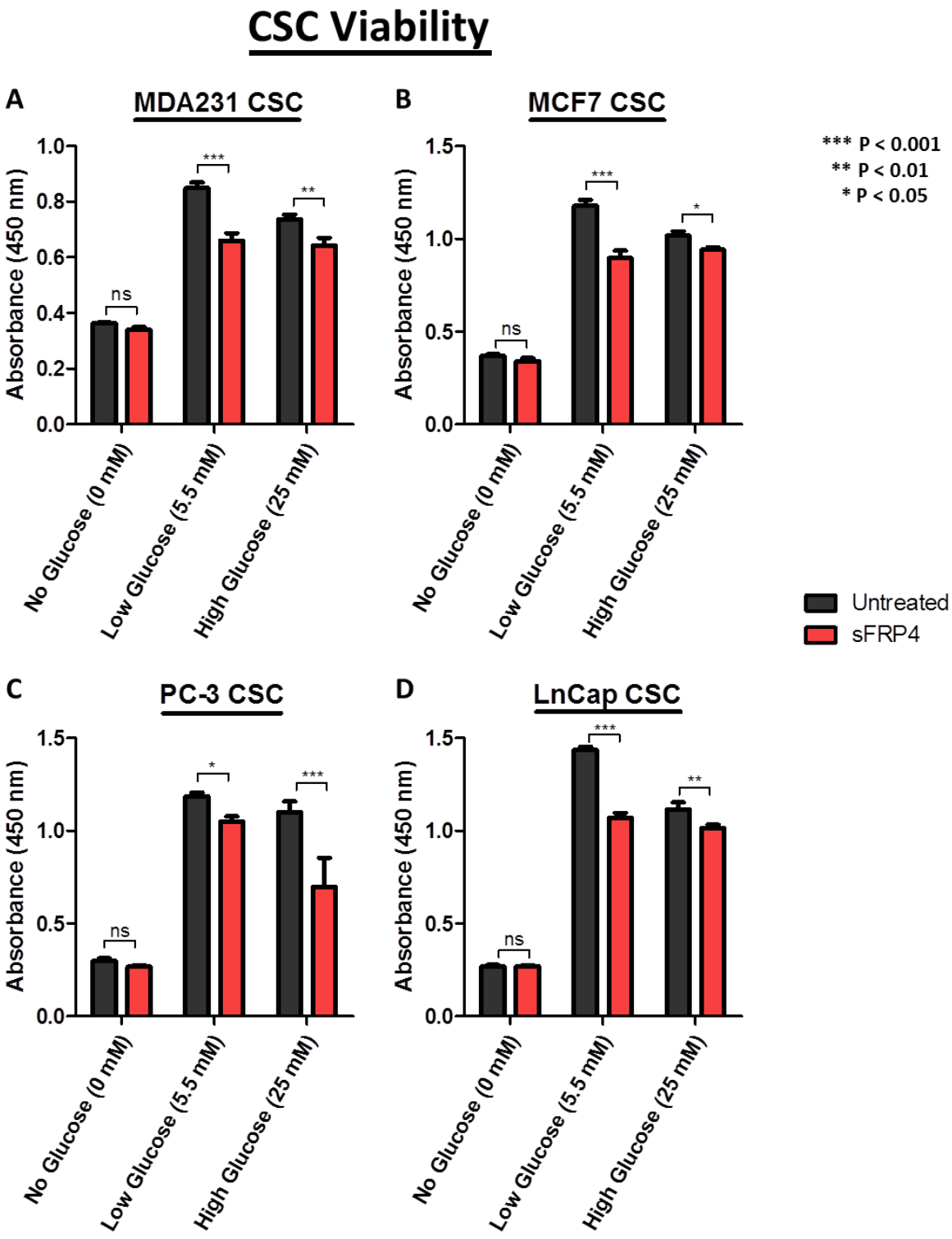
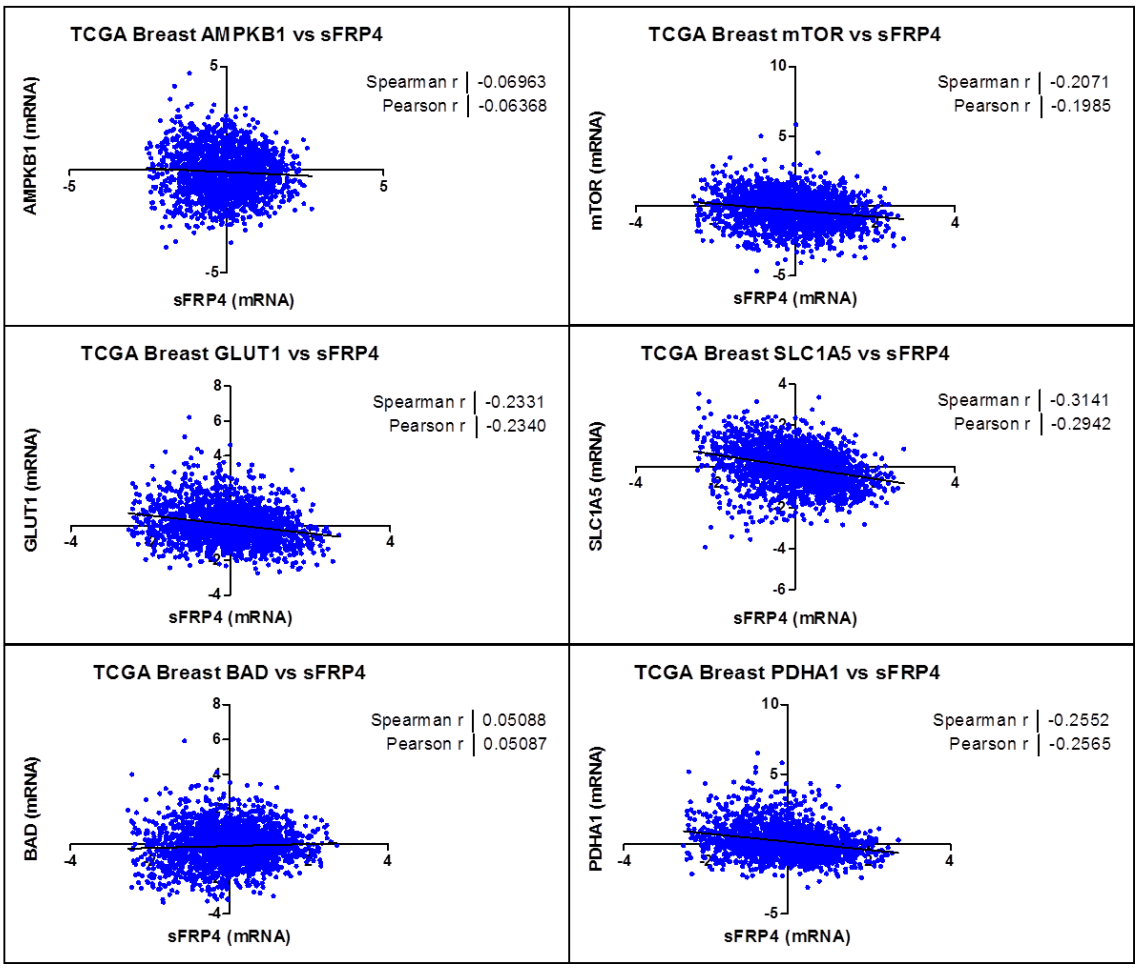


Figure 2: The effect of sFRP4 on CSC viability: Viability assays were performed using CCK-8 after treatment of CSCs derived from A) MDA231; B) MCF7; C) PC-3; and D) LnCap cell lines treated with sFRP4 for 24 h. Statistical analysis was performed using ANOVA for analysis variance with Bonferroni test for comparison showing significance as ***P<0.001; **P<0.01; *P<0.05. Data are mean ± standard error of the mean from 3 independent experiments.

5.3.3 Correlation coefficient of sFRP4 with metabolic gene-set

Our initial goal was to generate a gene-expression signature representing sFRP4 activation and to use that signature to identify metabolic targets in breast and prostate cancer through The Cancer Genome Atlas [27]. The set of genes were further narrowed by identifying the subset of genes with established or putative roles in cancer metabolism. Because some of the genes were identified as cancer amplified genes, we further analyzed the gene set to identify putative metabolism driver genes. This was done by calculating the Spearman and Pearson correlation coefficient between the mRNA expression values from The Cancer Genome Atlas (TCGA) patient tumour data. Correlation coefficients were calculated for both breast (Figure 3A) and prostate (Figure 3B) cancer, and average correlations were calculated for each gene. The gene set comprised metabolic genes such as *AMPKB1* (AMP Kinase), *mTOR* (Mammalian target of rapamycin), *GLUT1* (glucose transporter), *SLC1A5* (Glutamine transporter), *BAD* (Bcl-2 associated death promotor), and *PDHA1* (Pyruvate dehydrogenase). The analysis revealed a negative correlation coefficient between sFRP4 and the gene set, indicating an inhibitory effect of sFRP4 of all those genes. The only gene to have a positive correlation coefficient was *BAD*, indicating the pro-apoptotic capacity of sFRP4 in cancer metabolism in both the tumours examined. Although *AMPK* showed a minimal correlation, it plays an important role in regulating the PI3K/ AKT/ mTOR signalling cascade, and we decided to include this gene set for our protein modification study.

A


TCGA Breast AMPKB1 vs sFRP4
Spearman r | -0.06963
Pearson r | -0.06368
AMPKB1 (mRNA)
sFRP4 (mRNA)
TCGA Breast mTOR vs sFRP4
Spearman r | -0.2071
Pearson r | -0.1985
mTOR (mRNA)
sFRP4 (mRNA)
TCGA Breast GLUT1 vs sFRP4
Spearman r | -0.2331
Pearson r | -0.2340
GLUT1 (mRNA)
sFRP4 (mRNA)
TCGA Breast SLC1A5 vs sFRP4
Spearman r | -0.3141
Pearson r | -0.2942
SLC1A5 (mRNA)
sFRP4 (mRNA)
TCGA Breast BAD vs sFRP4
Spearman r | 0.05088
Pearson r | 0.05087
BAD (mRNA)
sFRP4 (mRNA)
TCGA Breast PDHA1 vs sFRP4
Spearman r | -0.2552
Pearson r | -0.2565
PDHA1 (mRNA)
sFRP4 (mRNA)

B

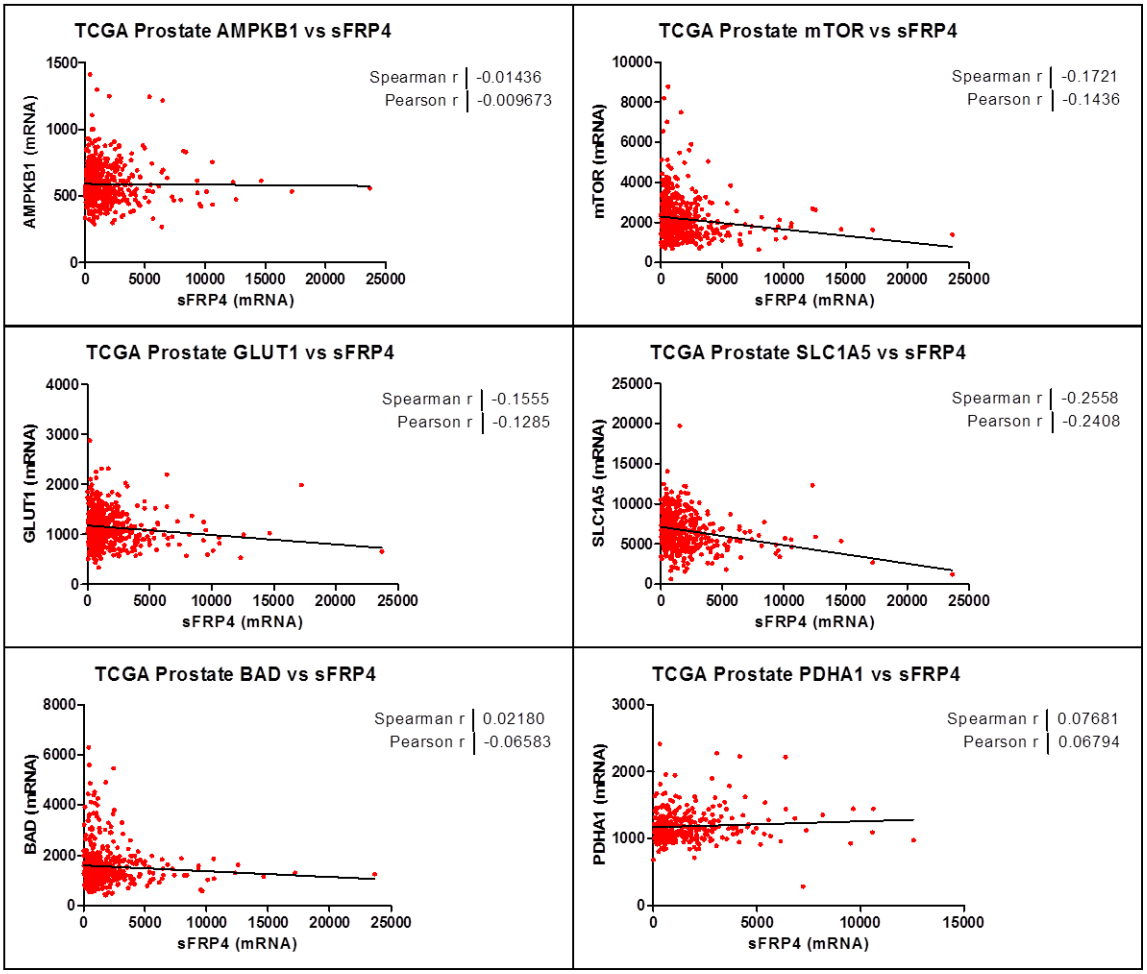


Figure 3: The correlation coefficient of sFRP4 with metabolic gene-set: Spearman and Pearson correlation coefficient between the mRNA expression values from TCGA patient tumour data. A) Breast Invasive Carcinoma (n=825) and B) Prostate Adenocarcinoma (n=333).

5.3.4 The effect of glucose and sFRP4 on glucose-uptake in CSCs

To better understand the effect of glucose on glycolytic metabolism, the CSCs were isolated in various glucose concentrations, and cellular uptake was detected with a Glucose Uptake-glo assay (Figure 4). Glucose induced an increase in glucose uptake by 2 folds in MDA231 CSCs, 5 folds in MCF7 CSCs and PC3 CSCs low glucose groups, whereas in the high glucose groups there was increase by 2 folds for MDA231 CSCs and LnCap CSCs, and 6 folds in PC3 CSCs, compared with the no glucose groups (Figure 4A, B, C, and D). Breast CSCs had higher glucose uptake in the low glucose groups (Figure 4A and Figure 4B); whereas prostate CSCs had higher glucose uptake in the high glucose groups (Figure 4C and Figure 4D). We then monitored the effect of sFRP4 on glucose uptake. The levels of glucose uptake in low glucose groups significantly increased in all CSCs, whereas they decreased significantly in high glucose groups, suggesting that sFRP4 has a direct effect on glycolytic flux and increasing the glycolytic activity.

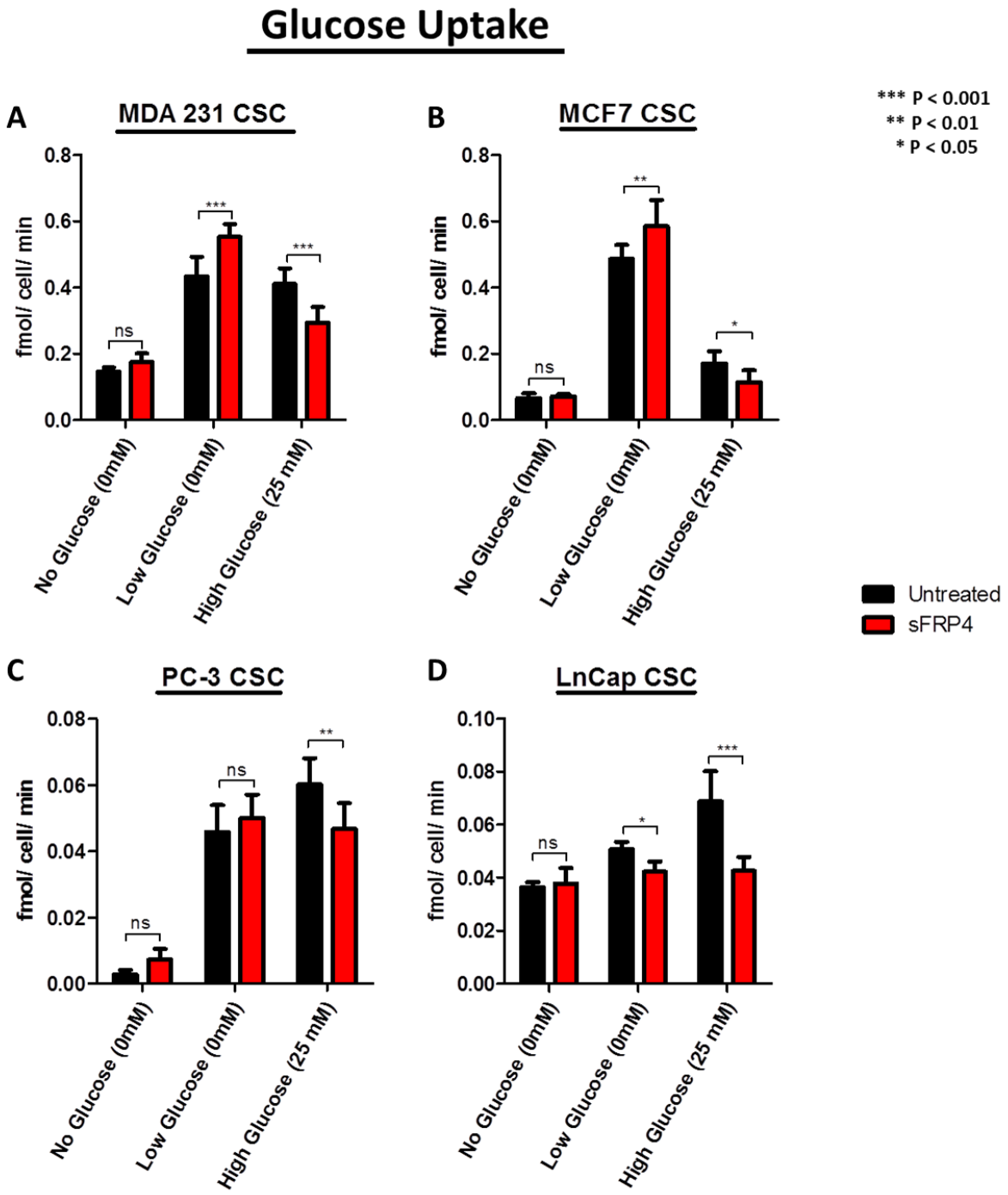


Figure 4: The effect of sFRP4 on glucose-uptake in CSCs: CSCs were isolated in various glucose concentrations and treated with sFRP4 (250pg) for 24 h. Cellular uptake was detected with Glucose Uptake-glo assay. A) MDA231 CSCs; B) MCF7 CSCs; C) PC-3 CSCs; and D) LnCap CSCs. Statistical analysis was performed using ANOVA for analysis variance with Bonferroni test for comparison showing significance as ***P<0.001; **P<0.01; *P<0.05. Data are mean ± standard error of the mean from 3 independent experiments.

5.3.5 Changes in extracellular metabolites with sFRP4 treatment of CSCs

To further characterize the metabolic profiles of CSCs, changes in extracellular metabolite levels during CSC growth in culture were measured. The starting concentration of glutamine is consistent in all CSCs medium, and as cells grow they consume these metabolites. During growth, glutamate is secreted and accumulated in the medium. The CSCs consumed glutamine and secreted glutamate with time and cell density dependence. Glutamine consumption was more robust in CSCs isolated in all glucose groups (Figure 5A, B, C, and D). The glutamine uptake increased with increasing glucose content in the medium. In contrast, glutamate secretion (Figure 5E, F, G, and H) was also observed in all CSCs, indicating glutaminolysis activity. The relative luminescence units (RLU) increased in glutamine uptake with increase in glucose content, indicating the co-activity of glycolysis and glutaminolysis. However, glutamate secretion was significantly different in all the CSCs. Upon addition of sFRP4, we observed a decrease in glutamine uptake and glutamate secretion in all CSCs. However, PC3 CSCs (Figure 5C and G) exhibited a marked effect following sFRP4 treatment, as we observed a higher inhibition of extracellular metabolite secretion.

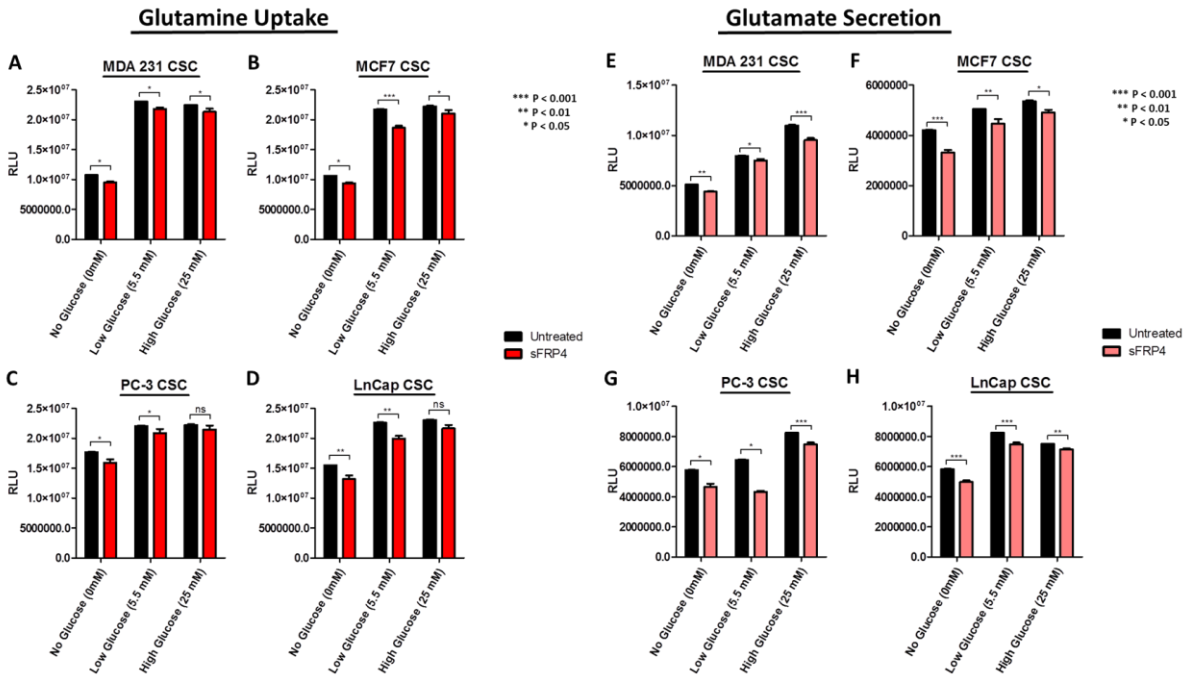


Figure 5: Extracellular metabolites with sFRP4 treatment of CSCs: Changes in glutamine uptake and glutamate secretion in CSCs grown in culture medium with increasing glucose concentrations were measured. A) MDA231 CSCs; B) MCF7 CSCs; C) PC-3 CSCs; and D) LnCap CSCs were treated with sFRP4 for 24 h. Statistical analysis was performed using ANOVA for analysis variance with Bonferroni test for comparison showing significance as ***P<0.001; **P<0.01; *P<0.05. Data are mean ± standard error of the mean from 3 independent experiments.

5.3.6 Changes in redox signature with sFRP4 treatment of CSCs

The nicotinamide adenine dinucleotide redox couple (NAD+/NADH) is a marker of catabolism. Here we aimed at describing the divergent redox profile of the CSC population isolated from breast and prostate tumour cell lines under different glucose concentrations. We also demonstrated the effect that sFRP4 has on the divergent redox signature of these CSCs (Figure 6). The results show the comparison of NAD+/NADH ratio in CSCs with no, low, and high glucose content, and the effect of sFRP4 in these conditions. The NAD+/NADH ratio was considerably higher in MDA231 CSCs, and sFRP4 treated CSCs showed a significant decrease (Figure 6A). The NAD+/NADH ratio in MCF-7 CSCs gradually decreased with an increase in glucose concentration, whereas sFRP4 had a minimal effect (Figure 6B). In PC3 CSCs, the NAD+/NAD ratio had an inverse activity as compared to MCF7 CSCs; here the ratio increased with an increase in glucose concentration, and sFRP4 treatment oscillates CSC catabolism by increasing the ratio in the no-glucose group, whereas it decreased the ratio in low and high-glucose groups (Figure 6C). The NAD+/NADH ratio in LnCap CSCs followed this trend, and decreased the redox activity as the glucose concentration increased; moreover, sFRP4 had no aberrant effect and decreased the ratio in 2 out of 3 glucose groups (Figure 6D). The generalised observation was that hormone-independent CSCs such as MDA231 (ER-) and PC3 (AR-) demonstrated a higher NAD+/NADH ratio with increasing glucose concentrations (Figure 6A and C); whereas hormone-dependent CSCs MCF7 (ER+) and LnCap (AR+) demonstrated a decrease in the NAD+/NADH ratio with an increase in glucose concentration (Figure 6B and D).

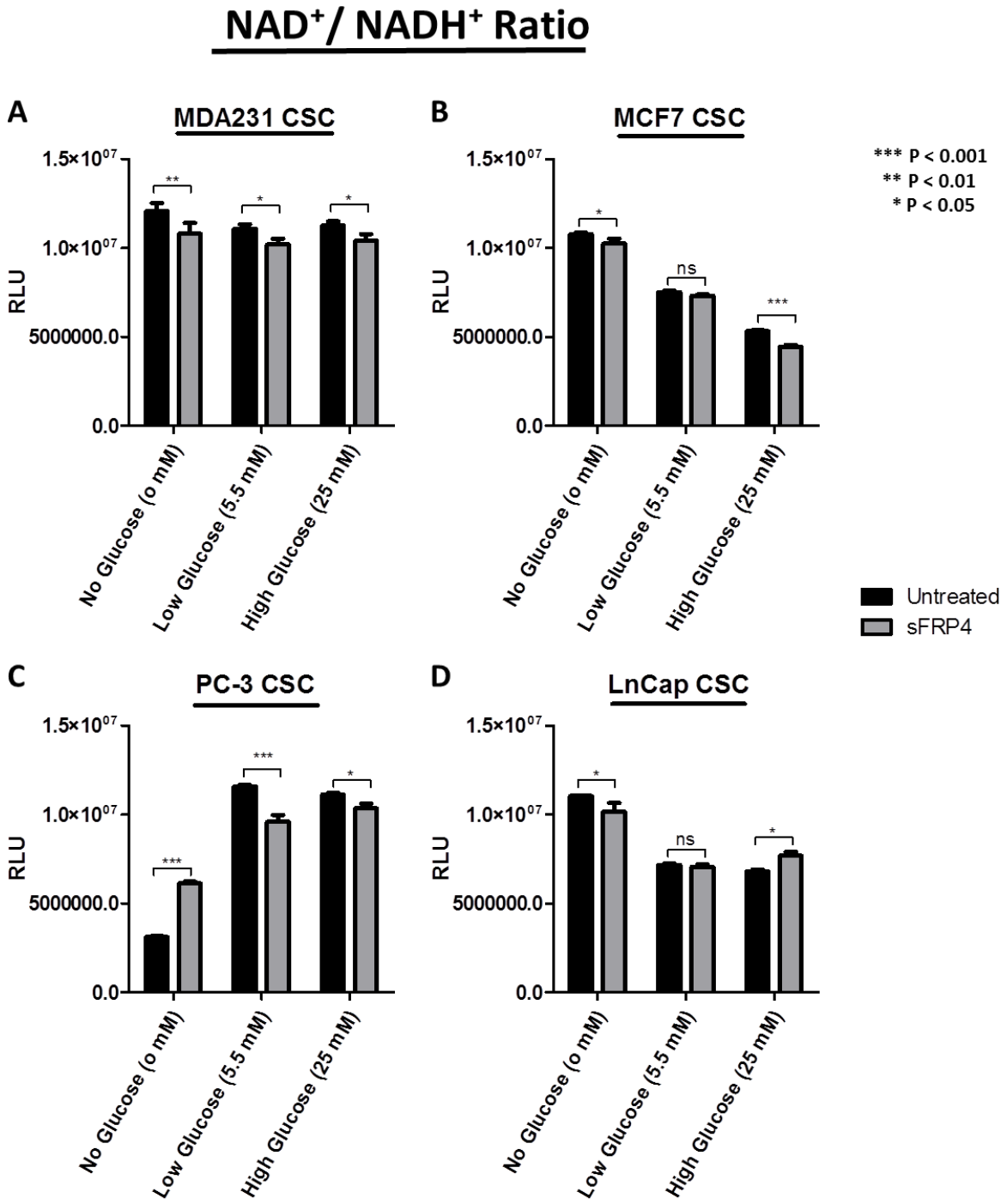
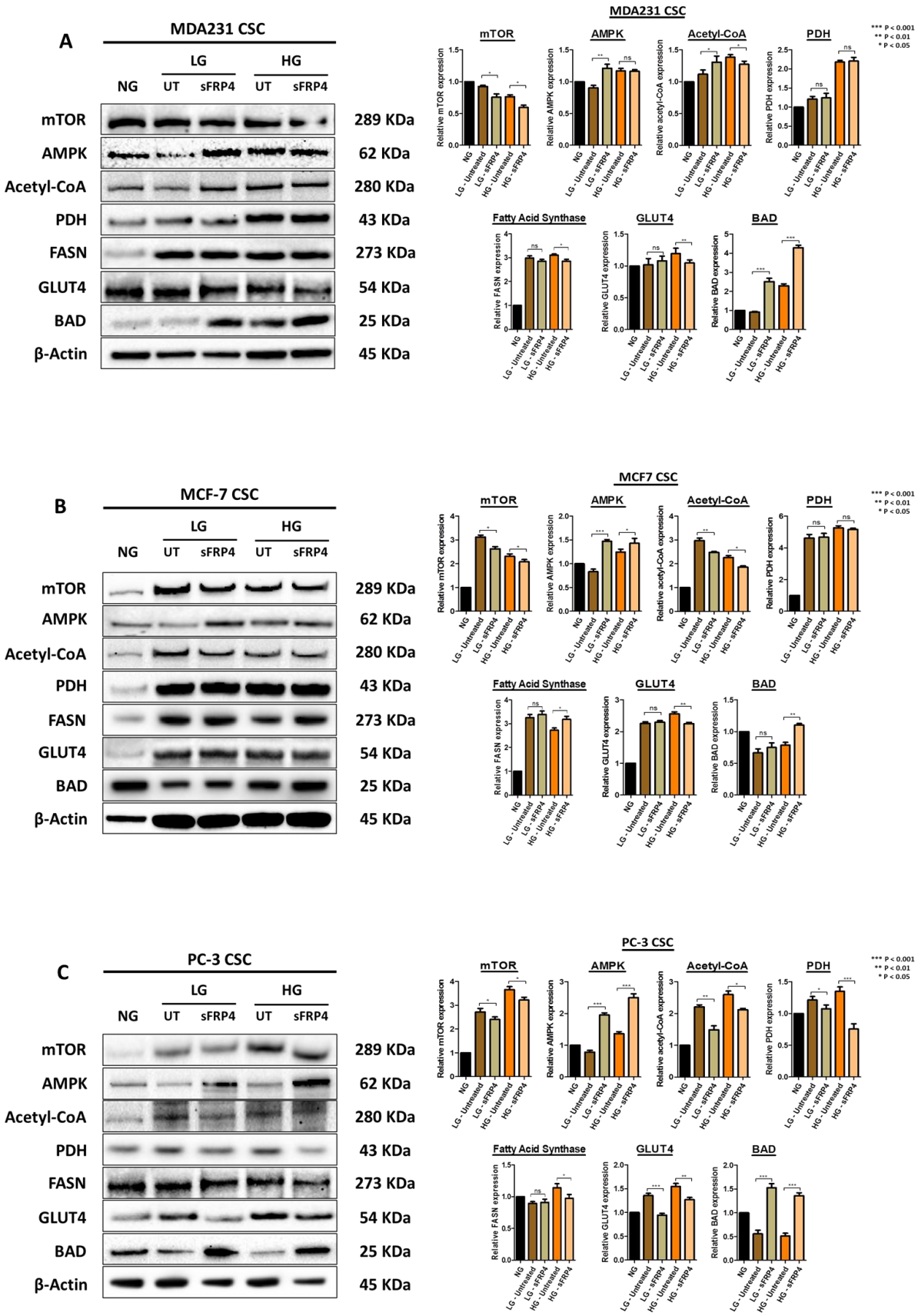


Figure 6: Redox signature with sFRP4 treatment of CSCs: Comparison of NAD+/NADH ratio in CSCs with no, low, and high glucose content. A) MDA231 CSCs; B) MCF7 CSCs; C) PC-3 CSCs; and D) LnCap CSCs were treated with sFRP4 for 24 hr. Statistical analysis was performed using ANOVA for analysis variance with Bonferroni test for comparison showing significance as ***P<0.001; **P<0.01; *P<0.05. Data are mean ± standard error of the mean from 3 independent experiments.

5.3.7 The effect of sFRP4 on CSC metabolism target proteins.

Following CSC isolation in different glucose concentrations and sFRP4 treatment, we investigated the post-translational modifications in CSCs for central regulator of cell metabolism (mTOR), AMP-activated protein kinase (AMPK), rate-limiting enzyme (Acetyl-CoA Carboxylase), metabolic oncogene (Fatty acid synthase), metabolic gatekeeper (Pyruvate dehydrogenase), glucose transporter (GLUT4), and Bcl-2 associated death promotor (BAD). MTOR was highly expressed in all the untreated groups but decreased when treated with sFRP4 in low and high glucose groups, except for the LnCap CSC high glucose group (Figure 7D). The AMPKα protein levels were observed in all the glucose groups and were significantly elevated with sFRP4 treatment, except the MDA231 CSC high glucose group (Figure 7A). This indicates the AMPKα activity in regulating mTOR, where AMPK phosphorylates mTOR to suppress the PI3K/ AKT/ mTOR signalling cascade. The protein levels of Acetyl-CoA Carboxylase decreased in all CSC high glucose groups following sFRP4 treatment, whereas there was an increase post-sFRP4 treatment in the MDA231 CSC low glucose group (Figure 7A). Overexpression of pyruvate dehydrogenase confers higher pyruvate conversion to acetyl CoA; however, sFRP4 treatment significantly decreased protein expression in PC3 CSCs for all glucose groups (Figure 7C) and LnCap CSC low glucose groups (Figure 7D). With sFRP4 treatment, no change in PDH was observed in breast CSCs, though in untreated CSCs there was an increase in PDH expression with an increase in glucose concentration (Figure 7A and B). There was a reduction in fatty acid synthase (FASN) protein expression following treatment with sFRP4 in high-glucose groups except LnCap CSCs; however, there was a minimal effect in all the CSCs in low-glucose groups except LnCap CSCs, and there was no expression seen in the LnCap CSC no-glucose group (Figure 7D). The glucose transporter GLUT4 protein expression in all CSCs increased with an increase in glucose concentration; however, GLUT4 decreased with sFRP4 treatment in all CSCs in high glucose group, and only the PC3 CSCs low-glucose group exhibited a decrease in GLUT4 expression post-sFRP4 treatment (Figure 7C). LnCap CSCs were non-responsive to sFRP4 treatment on GLUT4 (Figure 7D). Expression of Bcl-2 associated death promotor BAD was lower in untreated CSCs but increased significantly with sFRP4 treatment across all glucose groups. The increased BAD protein expression confirms the pro-apoptotic role of sFRP4.


A
MDA231 CSC
LG
HG
NG
UT
sFRP4
UT
sFRP4
mTOR
AMPK
Acetyl-CoA
PDH
FASN
GLUT4
BAD
β-Actin
289 KDa
62 KDa
280 KDa
43 KDa
273 KDa
54 KDa
25 KDa
45 KDa
MDA231 CSC
mTOR
AMPK
Acetyl-CoA
PDH
Fatty Acid Synthase
GLUT4
BAD
Relative mTOR expression
Relative AMPK expression
Relative acetyl-CoA expression
Relative PDH expression
Relative FASN expression
Relative GLUT4 expression
Relative BAD expression
NG
LG - Untreated
LG - sFRP4
HG - Untreated
HG - sFRP4
*** P < 0.001
** P < 0.01
* P < 0.05
ns
B
MCF-7 CSC
MCF7 CSC
C
PC-3 CSC
PC-3 CSC

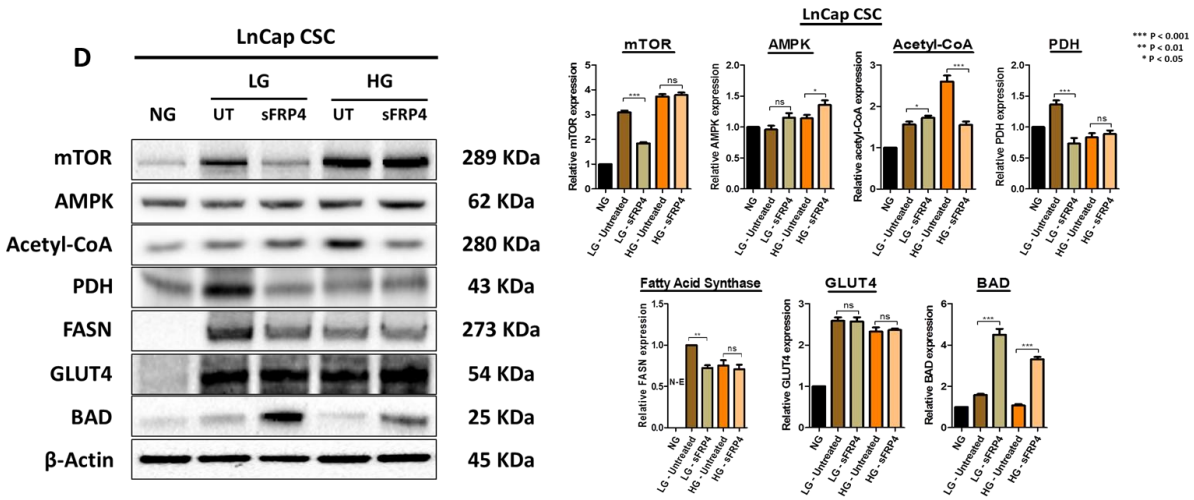


Figure 7: The effect of sFRP4 on CSC metabolism target proteins: Changes in CSC metabolic profile and the post-translational modifications with increasing glucose conditions. A) MDA231 CSCs; B) MCF7 CSCs; C) PC-3 CSCs; and D) LnCap CSCs were treated with sFRP4 for 24 h. Densitometry analysis was performed using ANOVA for analysis variance with Bonferroni test for comparison showing significance as ***P<0.001; **P<0.01; *P<0.05. Blots and relative protein expressions are mean ± standard error of the mean from 3 independent experiments.

5.4 Discussion

It has been established for decades that cancer cells actively use glycolytic metabolism even in the presence of oxygen, which is known as the Warburg effect [14, 15]. The cancer cells are benefited by the high glycolytic rate for ATP production and other metabolites [29]. Due to this prevalent glycolytic elevation in cancer cells and its clinical relevance, such metabolic alterations have been considered as the hallmark of cancers [30]. Recent studies suggest that CSCs may have even more active glycolytic activity compared to the bulk of tumour cells [11, 13, 31]. Consistently, the current study showed the CSCs to be highly glycolytic, and these observations underscore the importance of glucose as an essential nutrient for CSCs and suggest the possibility that levels of glucose in the tumour microenvironment might significantly affect CSC survival. Therefore, the knowledge of CSC metabolism is of great importance for our understanding of reproductive tumours (e.g. breast and prostate tumours), which are tumours with poor prognosis. To gain further insights into CSC metabolism, we investigated the role of sFRP4 on CSCs isolated in culture medium with various glucose concentrations. Our study demonstrates sFRP4 elicits an anti-proliferative effect, spheroid disruption, decreases glucose uptake, glutamine uptake, glutamate secretion, redox signature, and the signalling cascade responsible for cell survival, and promotes apoptosis within the CSCs, therefore indicating sFRP4`s potential role in regulating CSC metabolism.

While important roles of tumour tissue niches in affecting CSCs have gained attention in recent years, the impact of key nutrients in the tumour microenvironment remains largely unknown. The CSCs niches preserve the CSCs' phenotypic plasticity, facilitate metastatic potential, and support high expression of drug efflux transporters, making them highly chemo-resistant [32]. In this study, we used various functional assays to evaluate the effect of glucose and sFRP4 on CSCs, and used Western blot analyses to investigate the underlying mechanisms. Our results exemplify that glucose plays a major role in promoting the CSC phenotype (Figure 1). We also showed that targeting the Wnt signalling pathway by using sFRP4 has the capacity to disrupt the CSCs niches in various glucose concentrations (Figure 1). CSCs in a glucose depleted medium were morphologically stressed and more susceptible to sFRP4. Spheroid disruption by sFRP4 decreases the CSCs' plasticity and cell-cell adhesion,

initiating the CSCs' differentiation towards tumour cells and reducing their self-renewal capacity. The induction of CSCs by glucose appears to be a reversible phenomenon. As shown in Figures 1 and 2, switching CSCs from a no glucose medium to a low glucose or high glucose medium led to an increase in their viability and they became morphologically more robust. Consistently, glucose deprivation caused rapid depletion in CSC viability, which re-appeared when glucose was replenished.

In previous studies, our group has shown that sFRP4 has an anti-proliferative capacity in CSCs derived from breast, prostate, ovary, glioblastoma multiforme, and head and neck tumours [33, 34, 8]. In this study we demonstrated that sFRP4 decreased the viability of CSCs in increasing glucose concentrations when compared to CSCs in a glucose depleted medium (Figure 2), indicating sFRP4s' anti-proliferative capacity is independent of exogenous key nutrients in the microenvironment.

Most cancer cells rely more on glycolysis rather than on oxidative phosphorylation for glucose metabolism [14]. The active utilization of glucose by tumour cells constitutes the basis of 2-[^{18}F]fluoro-2-deoxy-$_{D}$-glucose positron emission tomography (^{18}FDG-PET) imaging for cancer diagnosis, and positive FDG-PET signals post-treatment predict poor prognosis [35-38]. CSCs are chemoresistant cells, and it is possible that with conventional chemotherapeutic treatment the residual lesions within the tumour would enrich for CSCs with elevated glycolytic activity. In a previous study, CSCs were isolated from human non-small cell lung carcinoma (NSCLC) and colon cancer cell lines [39]. Using flow cytometry, they compared the glucose metabolic activity between CSCs and non-CSCs; glucose uptake was significantly increased in CSCs compared to non-CSCs, indicating that CSCs were more glycolytic than their normal counterparts. To better understand the effect of glucose on glycolytic metabolism, we isolated CSCs in glucose deprived medium and increasing concentrations of glucose. We observed high glycolytic activity within CSCs isolated in low (5.5 mM) glucose medium, and comparatively less in high glucose (25 mM) medium. Meanwhile, we also investigated the effect of sFRP4 on glucose uptake, where we observed varying effects. We postulate that sFRP4 had stressed the CSCs in the glucose deprived medium, driving them to initiate higher glycolytic activity; whereas CSCs in high glucose medium showed an inhibitory effect on glucose uptake, suggesting that sFRP4 has a direct effect on glycolytic flux. Furthermore, the glucose uptake was variable in all CSCs, and no

trend was observed. Other studies have also found that there is no differential response to epidermal growth factor (EGF) and basic fibroblast growth factor (FGF) in the media used to isolate CSCs, ruling out any interference of these growth factors on CSCs glucose uptake [40]. Although the presence of glucose in CSC culture medium has been shown to significantly increase the viability of CSCs in the overall isolation process, glucose uptake is an essential process and a key nutrient for CSCs [39]. A recent proteomic and targeted metabolomics analysis between breast CSCs and their counterpart revealed a metabolic pathway associated with the stem-like conditions, indicating that breast CSCs shift from mitochondrial OXPHOS towards fermentative glycolysis [41].

Although cancer cells exhibit high rates of glycolysis, their mitochondrial OXPHOS remains intact and becomes progressively more dependent on glutamine metabolism [42]. In cancer cells, the rate of glutamine conversion to lactate is higher compared to normal cells, which represents an alternative metabolic pathway to glucose consumption in a glucose depleted microenvironment [43]. Glutaminase converts glutamine to glutamate through glutaminolysis. Glutaminase and glutamine levels in the cell culture medium correlate with the cancer cell proliferation, whereas glutamate levels are associated with tumour aggressiveness [44]. Moreover, glutamine's function to promote cell growth is widely dependent on the epigenetic background of the tumour [45, 46]. Glutamine depletion induces apoptosis in melanoma and prostate cancer cells, and using acivicin to inhibit glutaminolysis has been very effective in animal models of cancer [47-49]. In addition, another study demonstrated the targeting of glutamine uptake as a new therapeutic strategy to treat acute myeloid leukaemia [50]. However, glutamine deprivation of CSCs is less well characterized. We demonstrated in our study that sFRP4 significantly decreased the glutamine uptake and glutamate secretion in all CSC glucose groups, indicating the key role of sFRP4 in glucose/glutamine metabolism. Another study demonstrated that glutamine promotes cell growth in ovarian cancer cells by activating the mTOR/S6 and MAPK pathways [51]. This enables us to suggest that targeting glutaminolysis by sFRP4 might prove a valuable step in regulating CSC metabolism.

The NAD+/NADH ratio is directly impacted by glycolytic and mitochondrial activities that change during metabolic reprogramming. The NAD+/NADH redox state plays a key role in cancer cell stemness [52]. Nicotinamide, the NAD precursor, protects cells from apoptosis

and senescence by accelerating cell proliferation and alleviating oxidative stress. Accordingly, in CSCs, increased glucose metabolism reduces the level of reactive oxygen species (ROS) to promote EMT [10, 53], whereas the level of NADH is decreased with a decrease in the ratio of reduced glutathione (GSH) to oxidised (GSSH) glutathione [54]. A high NADH level is a property that is conserved between normal and cancerous stem cells [55]. A previous study has also demonstrated that when CSCs are fed with mitochondrial fuel (L-lactate or ketone bodies), CSCs quantitatively produce more NADH in response to the stimulus compared to non-CSCs [56]. In addition, NAD+ depletion, using the NAMPT inhibitor FK866, potently blocked spheroid formation [55]. We demonstrated a significant reduction in the NAD+/NADH ratio in all CSCs post-sFRP4 treatment, suggesting that a higher NAD+ content is important for enhancing the resistance to stress induced by ROS in CSCs; whereas a decreased NAD+/NADH ratio makes CSCs more susceptible to reprogramming their redox state.

There is growing evidence on the role of the mTOR pathway in the maintenance of CSCs. Prostate cancer radio-resistance is associated with EMT and enhanced CSC phenotypes via activation of the PI3K/Akt/mTOR signalling cascade [57]. Activation of the mTOR signalling pathway enhances breast CSC colony formation ability *in vitro* and tumorigenicity *in vivo* [58]. Suppression of mTOR decreases ALDH1 activity in colorectal CSCs [59, 60]. In glioblastoma CSCs, cross-inhibitory regulation between the MEK/ERK and PI3K/ mTOR signalling cascade contributed to self-renewal and tumorigenic capacity [61]. Aberrant activation of the PI3K/Akt/mTOR signalling pathway leads to an increase in chemokine (C-X-C motif) receptor 4 (CXCR4), which corresponds to maintenance of stemness in NSCLC cells [62]. Interestingly, metformin decreased radio-resistance of CSCs in a mouse fibrosarcoma cells and MCF7 breast cancer cells by activating AMP-activated protein kinase and suppressing mTOR expression [63]. We demonstrated that sFRP4 decreases mTOR protein expression and activates AMP kinase (AMPK), which in turns inhibits the PI3K/Akt/mTOR signalling cascade via phosphorylation of mTOR. It is possible that the anti-tumour activity of sFRP4 *in vitro* maybe associated with inhibition of the insulin/IGF-1 pathway through AMPK activation. AMPK regulates mTOR activity through activation of the tuberous sclerosis protein 1/2 complex [64, 65].

In addition to glucose and glutamine, fatty acids are an important energy source incorporated in extracellular media, or can be obtained endogenously by accumulating lipid droplets [66]. Fatty acid synthesis is an anabolic process, which starts with converting acetyl CoA to malonyl CoA by acetyl CoA carboxylase. We found a higher expression of fatty acid synthase (FASN) in breast CSCs for all glucose groups. Notably, a high expression of FASN has been linked to poor prognosis of pancreatic ductal adenocarcinoma patients and depends heavily on induction of EGFR/ERK signalling [67]. Furthermore, FASN promotes EMT in ovarian [68], breast [69] and colorectal [70] cancers. Inhibition of FASN leads cancer cells to apoptosis, mainly by inhibiting DNA replication and the production of anti-apoptotic proteins [71]. We saw a minimal effect of sFRP4 on FASN, although breast CSCs in the high glucose groups responded with a decrease in FASN, and the LnCap CSC low glucose group had a maximal decrease in FASN. We propose that sFRP4's effect is associated with the glycolytic switch of CSCs occurring in different glucose concentrations.

Pyruvate dehydrogenase (PDH) is a key enzyme that mediates the entry of pyruvate to mitochondria where it facilitates its conversion to acetyl CoA. PDH activity is regulated by pyruvate dehydrogenase kinase 1 (PDK-1) [72]. In CSCs, PDK-1 via the TCA cycle, phosphorylates pyruvate dehydrogenase and suppresses the pyruvate to acetyl-CoA conversion. Furthermore, suppressing the metabolic flow of pyruvate in mitochondria induces the conversion of pyruvate to lactate in the cytosol [39, 73]. We observed a decrease in acetyl CoA expression in CSCs after treatment with sFRP4, indicating the negative effect of sFRP4 on the mitochondrial membrane potential. Higher PDH expression was observed in CSCs in the high glucose group, and sFRP4 had an impairing effect on PDH expression in prostate CSCs. PC-3 CSC treatment with sFRP4 decreased PDH expression in both glucose groups. A recent study revealed that chemical inhibition (via soraphen A) of acetyl CoA carboxylase suppresses self-renewal growth of CSCs derived from the MCF7 cell line [74]. However, the conversion of pyruvate to acetyl CoA in CSCs is still unclear, and how glucose concentration might influence the process is still something worth exploring.

Tumorigenesis is associated with enhanced cellular glucose uptake and increased metabolism. The transmembrane glucose transporter (GLUT) proteins mediate glucose uptake in cancer cells, and initiate the glucose utilisation cascade [75]. GLUT4 is aberrantly expressed in many tumours, though no study has been undertaken within CSCs. We

demonstrated that GLUT4 is expressed in all CSCs, and there is increased expression with an increase in glucose concentration. Furthermore, we also showed the inhibitory effect of sFRP4 on GLUT4 expression.

Most anti-cancer drugs exert their effect through triggering the apoptosis pathway, although CSCs escape apoptosis by altering their expression levels of pro-apoptotic and anti-apoptotic Bcl-2 family members [8]. BAD (Bcl-2 associated death promoter) is a member of the Bcl-2 family that, when dephosphorylated, initiates apoptosis by heterodimerizing with anti-apoptotic proteins Bcl-xl and Bcl-2 (15). *In vivo*, BAD phosphorylation was detected in CSCs of 83% breast cancer biopsies [76]. The overexpression of BAD is correlated with chemoresistance. Interestingly, high-grade tumours exhibit higher BAD protein levels than those with low-grade cancer, suggesting a role in tumour progression [77]. We demonstrated a gradual increase in (dephosphorylated) BAD expression in CSCs treated with sFRP4. Moreover, sFRP4 treatment elevated BAD in all glucose groups. The increased expression of dephosphorylated BAD is an indicator for apoptosis, and an increased expression depicts the activation of caspase cleavage [78]. BAD expression was consistently high in all CSCs treated with sFRP4. The elevated expression of apoptotic proteins within all the CSCs reinforces sFRP4's role as a pro-apoptotic agent.

In summary, sFRP4 plays an important role in breast and prostate CSC metabolism by reducing the CSCs' proliferative capacity and glucose uptake, modulating their redox signature, and decreasing the CSC's survival signalling cascade by targeting the mTOR complex, making them more responsive to therapy. Further *in vivo* studies may confirm the efficacy of sFRP4 in altering CSC metabolism to prevent tumour relapse and lead to tumour resolution.

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Chapter 6

6 Discussion and Conclusion

6.1 Preface:

The Wnt signalling pathway is a central pathway in both development and homeostasis that regulates cell proliferation, survival, and progression. Aberrant activation of the Wnt pathway can result in neoplasia and cancer. It has been observed that WNT/β-catenin signalling is important for proliferation and self-renewal of cancer stem cells due to its multiple genetic alterations in association with human tumorigenesis; silencing these pathways and treating them with sFRP4 could result in improving the chemo-response. This chapter brings together key findings from chapter 2 to 5 inclusive, summarising the new understanding we have uncovered as a novel approach of treating CSCs with sFRP4 and would demonstrate the cell population's response epigenetically and metabolically. In addition, the WNT/β-catenin signalling pathway also contributes to CSC biology and chemo-resistance.

6.2 Introduction

The results presented in this thesis have demonstrated the association between aberrant Wnt signalling and cancer stem cells (CSCs). We have discovered that treatment of CSCs with sFRP4 would enable their chemo-sensitisation and improve chemotherapeutic efficacy, and will also provide the feasibility of using sFRP4 to inhibit CSC survival in order to induce metabolic reprogramming. We also demonstrated the possible involvement of methylation-mediated silencing of the sFRP gene family in CSCs, which during tumour development are hypermethylated, causing their transcriptional silencing. They key outcomes from this thesis are discussed below in further detail.

6.3 Key Outcomes

6.3.1 Characterisation of CSCs derived from Breast, Prostate, and Ovary tumours.

The drug resistance in CSCs is often correlated to the CSC surface marker profiles. We successfully characterized the CSCs in the form of spheroids for the expression of tumour-specific CSC markers $CD44^{+}/CD24^{-}$/low for breast CSCs, and $CD133^{+}/ CD44^{+}$ for prostate and ovarian CSCs, by using flow cytometry. The characterized CSCs were further used for functional analyses. A reduction in the expression of CSC markers was observed in our study, where we demonstrated that sFRP4 and sFRP4-derived peptides (SC301/SC401) in combination with chemotherapeutic drugs were able to decrease the $CD44^{+}/24^{-}$ population for breast-derived CSCs, and the $CD44^{+}/CD133^{+}$ population for prostate and ovary-derived CSCs. The studies described in chapter 2 and 3 confirm the success of sFRP4 and novel peptides in reducing CSC surface marker expression, indicating a breach of treatment-resistance.

6.3.2 Wnt antagonism disrupts CSC sphere forming capacity.

Clinically, CSCs reside in anatomically distinct regions within the tumour microenvironment (known as niches), which preserve the CSCs' phenotypic plasticity, facilitates metastatic potential, and supports high expression of drug efflux transporters, making them highly chemo-resistant. *In-vitro*, CSC niches are characterized by spheroid forming capacity in serum free conditions. In chapter 2 and 3 we have demonstrated that targeting the Wnt signalling pathway by using sFRP4 and sFRP4-derived peptides (SC301 and SC401) disrupts the niches when used in combination with chemotherapeutic drugs. Spheroid disruption by

Wnt antagonism decreases the CSCs' plasticity and cell-cell adhesion, initiating the CSCs' differentiation towards tumour cells and reducing their self-renewal capacity. This opens the gateway for chemotherapeutic drugs to target the cells at high potency. We confirmed spheroid disruption using immunofluorescence, and we observed the spheroid disruption was associated with a reduced CSC marker profile. We also showed that sFRP4 had the capacity to disrupt the CSC niches in various glucose concentrations. CSCs grown in glucose depleted medium were morphologically stressed and more susceptible to sFRP4, indicating that the role of sFRP4 is independent of extracellular nutrients within the microenviornment.

6.3.3 Wnt antagonist, sFRP4 reduces CSC Viability.

In chapter 2 and 3 we have demonstrated that sFRP4 and its peptides (SC301 and SC401) in combination with chemotherapeutic drugs decreased the viability of CSCs compared to drug treatment alone, indicating sFRP4's role in the increased chemo-response of CSCs. In chapter 5 we have demonstrated that sFRP4 decrease the viability of CSCs in increasing glucose concentrations compared to CSCs cultured in glucose depleted medium, indicating sFRP4's anti-proliferative capacity is independent of exogenous key nutrients in the microenvironment.

6.3.4 Wnt signalling regulates stemness in CSCs.

Genes controlling the stemness of CSCs have distinct functions and are important for CSC development and self-renewal, and are responsible for replicative quiescence. We assumed that cancer stemness inhibition can effectively suppress metastatic potential and tumour recurrence. We demonstrated that sFRP4 reduced the expression of various stemness genes including Sox2, Klf4, Nanog, and Oct4 when treated in combination with chemotherapeutic drugs. These genes encode key stemness transcription factors that are important for maintenance of pluripotency. These data demonstrated the role of sFRP4 in inhibiting CSCs by modulating stemness gene expression.

6.3.5 Aberrant Wnt signalling mediates apoptosis in CSCs.

In previous studies, the relationship between sFRP4 and apoptosis has been identified, demonstrating sFRP4 as a pro-apoptotic agent (1, 2). This was further confirmed by assessing the integrity of the mitochondrial membrane potential when the CSCs were

treated with sFRP4 and its peptides (SC301 and SC401). We further demonstrated the pro-apoptotic role with an elevation in caspase 3/7 expression in CSCs treated with sFRP4 and peptides (SC301 and SC401) alone or in combination with chemotherapeutic drugs, indicating the later onset of apoptosis. We also observed combinatorial treatment elevated Bax expression and inversed the effect on Bcl-xl. The Bax/Bcl-xl ratio is an indicator for apoptosis, and an increased ratio depicts the activation of caspase 3 (3). The Bax/Bcl-xl ratio was consistently high in all CSCs following combinatorial treatment. The elevated expression of apoptotic genes within all the CSCs indicates sFRP4's role as a pro-apoptotic agent. Furthermore, the sFRP4 treatment elevated BAD expression in all CSCs isolated in all glucose conditions. The increased expression of dephosphorylated BAD is an indicator for apoptosis. This confirmed that sFRP4 is independent of exogenous key nutrients in the microenvironment.

6.3.6 Wnt antagonism sensitizes CSCs.

In summary, sFRP4 and its peptides (SC301 and SC401) chemo-sensitize CSCs derived from breast, prostate, and ovary tumour cell lines by reducing their pro-oncogenic profile, which includes ABC drug transporters (ABCG2), cell survival protein (Cyclin D1), oncogenes (c-Myc), and the ability to escape cell death/apoptosis (Bcl-xl), while reducing their stemness capacity, production of cell survival protein (mTOR/ AMPK), and oncogene (FASN) expression, making them more responsive to chemotherapeutic drugs. The SC401 peptide was more potent in its activity compared to sFRP4 and SC301. Chemo-sensitization by these peptides *in vivo* may decrease the required chemotherapeutic load required to reduce the tumour mass. It would also prevent a sustained Wnt inhibition in order to provide a therapeutic window for chemotherapy while sparing normal Wnt-dependent tissues from the deleterious effects of a high chemotherapeutic load.

6.3.7 Loss of sFRP4 functions contributes to activation of Wnt signalling in cancer.

We showed loss of methylation within sFRP (1, 2, 3, 4, and 5) promotor regions after treatment with a DNMT inhibitor, and CSCs gaining an unmethylated promoter sequence after treatment consistently showed upregulation of sFRP mRNA expression. Therefore, the findings suggest that epigenetic processes mediated through methylation of 5`-CpG-islands in the promotor regions of sFRPs are involved in epigenetic alterations within CSCs derived from the tumour cell lines examined. Our protein analyses demonstrated increased sFRP4

expression, GSK3β expression, and phosphorylated β-catenin expression after demethylating treatment, which confirms our hypothesis that methylation, appears to functionally silence sFRP4 gene expression in CSCs. To further validate the relation between demethylation and inactivation of Wnt signalling, we analysed the important Wnt pathway-associated molecules, demonstrating a decrease in protein levels of active (unphosphorylated) β-catenin.

6.3.8 Activation of sFRP4 regulates CSC histone modifications.

In CSCs, the chromatins recruit DNMTs, resulting in *ex novo* methylation and gene silencing, and leading to silencing of tumour suppressor genes during tumour progression. Our findings substantiate the crosstalk between DNMT inhibition and histone modifications, exemplified by the decreased levels of EZH2 post sFRP4 treatment and upregulation of sFRP4 post DNMT1 inhibition. The sFRP4 treatment led to increased methylation of the H3K27me3 target and suppression of EZH2, highlighting the important role of EZH2 in self-renewal of CSCs. EZH2 plays an important role in CSCs, including repression of tumour suppressor genes (4-7), and β-catenin stabilization (8). As a consequence of the cellular changes induced by transcription and epigenetic alterations, the CSCs become dependent on EZH2 to preserve their self-renewal capacity (6). We also investigated the effect of sFRP4 activation on Histone H3 acetylation, as it is an important modification related to DNA repair, cell cycle regulation, and transcription activation (9). We observed that introduction of the pro-apoptotic sFRP4 led to acetylation in all CSCs, affecting their self-renewal capacity and tumour progression.

6.3.9 Changes in extracellular metabolites with sFRP4 treatment on CSCs

Most cancer cells rely more on glycolysis rather than on oxidative phosphorylation for glucose metabolism (10). Moreover, we investigated the effect of sFRP4 on glucose uptake. We propose that sFRP4 had stressed the CSCs cultured in glucose deprived medium, driving them to initiate higher glycolytic activity, whereas CSCs cultured in high glucose medium showed an inhibitory effect of glucose uptake, suggesting that sFRP4 has a direct effect on glycolytic flux. Meanwhile, the rate of glutamine conversion to lactate is higher in tumour cells compared to normal cells, which represents an alternative metabolic pathway to glucose consumption in a glucose depleted microenvironment (11). However, glutamine deprivation on CSCs is less well characterized. We demonstrated in our study that sFRP4

significantly decreased the glutamine uptake and glutamate secretion in the CSCs cultured under all glucose conditions, indicating the key role of sFRP4 in influencing glucose/glutamine metabolism.

6.3.10 Changes in redox signature with sFRP4 treatment on CSCs

The NAD+/NADH redox state plays a key role in cancer cell stemness (12). Nicotinamide, NAD precursor protect cells from apoptosis and senescence by accelerating the cell proliferation and alleviating oxidative stress. Accordingly, in CSCs increased glucose metabolism reduces the level of ROS to promote EMT (13, 14). We demonstrated significant reduction in NAD+/NADH ratio in all CSCs post-sFRP4 treatment, suggesting that higher NAD+ content is important for enhancing the resistance for stress induced by ROS in CSCs, whereas decreased NAD+/NADH ratio makes CSCs more susceptible to reprogram redox state.

6.4 Conclusion

Data presented in this thesis have demonstrated the association between aberrant Wnt signalling and CSCs. For the first time we have shown the role of sFRP4 in chemo-sensitisation, in basal glucose uptake, and involvement of methylation-mediated silencing of the sFRP gene family in CSCs derived from breast, ovary, and prostate cancer cell lines. We are also the first to report the sFRP4 regulation of CSC histone modification and CSC metabolism. Moreover, this is the first report demonstrating the role of sFRP4-derived novel peptides SC301 and SC401 on CSCs.

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To whom it may concern,

I **Abhijeet Deshmukh**, as a first author declare myself as the primary contributor to the experimental design, interpretation and writing to the paper/publication entitled "**Novel peptides derived from secreted frizzled-related protein 4 increase chemotherapeutic response of breast, prostate, and ovarian cancer stem cells".**

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Appendix 3

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Review article

Role of epigenetic modulation in cancer stem cell fate

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Chromatin immunoprecipitation

ABSTRACT

A sub-population of the tumor micro-environment consists of cancer stem cells (CSCs), which are responsible for the initiation and recurrence of cancer. Recently, epigenetic processes such as DNA methylation, histone modification, and chromatin remodeling have been found to be involved in inducing epigenetic factors in CSCs. Most of these processes, such as DNA methylation, generally occur in the genome that is rich in Cytosine-Guanine repeat sequences, also known as CpG islands, which are distributed throughout the human genome. The Polycomb gene (PcG) complex is a chromatin modifier facilitating the maintenance of embryonic and adult stem cells. Recent evidence suggests that the PcG is also involved in maintaining CSC stemness. We have presented various aspects and examples of how epigenetic modulation may drive or promote tumorigenesis and metastasis by alteration of key transcriptomic programs and signaling pathways in CSCs.

1. Introduction

The cancer microenvironment consists of some highly active cells that possess stem cell-like properties including high proliferation rate and differentiation; hence they have been referred to as cancer stem cells (CSCs) (Schatton et al., 2008). Some of the unique properties of CSCs include self-renewal and altered response to stress conditions (Blanco et al., 2016; Dayem et al., 2010). CSCs are a subset of the tumor cell population that possess high regenerative characteristics, metastatic capacity, sphere forming ability, and high ABC drug efflux transport systems (Adams and Strasser, 2008; Bao et al., 2012). CSCs help maintain the cancer environment by continuously differentiating into cancer cells; however, their origin is still a subject of controversy. Just like other adult stem cells in mammals, CSCs are also found to be arrested in a quiescent state, i.e. G0 phase (Cheong et al., 2003; Fukada, 2011). This state is however, reversible, by differentiation of CSCs into non-stem cancer cells that can further divide (Cheung and Rando, 2013). Therefore, recent studies have focussed on targeting CSCs to reduce the tumor bulk in order to eradicate cancer (Chen et al., 2013).

CSCs are enriched with various growth factors including epidermal growth factor (EGF) and fibroblast growth factor (FGF) that are involved in maintaining their stem-cell properties (Fillmore et al., 2010; Reynolds and Weiss, 1996). CSCs possess 4 transcription factors that help in maintaining the stem cell-like properties, which are Octomer-binding transcription factor 4 (Oct4) (Kim and Nam, 2011), Nanog homebox (NANOG) (Shan et al., 2012), Krupple-like factor 4 (Klf4) (Yu et al., 2011), and Sex determining region Y box-2 (Sox2) (Boumahdi et al., 2014). CSCs also express specific surface markers, for example – CD44 is the most common CSC surface marker for colon, gastric, breast, pancreatic, ovarian, and head/neck cancer; thus it is being targeted for cancer treatment therapies. Various examples of CSC surface markers are as listed in Table 1.

2. Epigenetics in CSCs

CSCs undergo similar genetic processes to normal stem cells, such as DNA methylation and chromatin remodeling. To understand the processes of self-renewal, differentiation, and proliferation in CSCs, the molecular pathways associated with epigenetics and gene regulation have been extensively examined (Clarke, 2005; Ravasio et al., 2016). DNA methyltransferase 1 (DNMT 1) regulates DNA methylation in human genes and is further maintained by DNMT3A and DNMT3B (Liao et al., 2015). In a study involving a leukemic murine model, wild-type mice with DNMT1 overexpression developed leukemia while DNMT1 knock-out mice ($DNMT1^{-/-}$) were free from disease (Bestor and Ingram, 1983; Chedin, 2011). Loss of DNMT1 expression resulted in

Abbreviations: CSCs, cancer stem cells; PcG, polycomb gene; PRC, polycomb repressive complex; CpG, *5′-C-phosphate-G-3′*; DNMT, DNA methyltransferase; EZH2, Enhancer Zeste Homologue 2; Hh, Hedgehog; MSP, methylation specific PCR; HAT, histone acetyltransferase; HDAC, histone deacetylase; HMT, histone methyltransferases; HDM, histone demethylases; ChIP, chromatin immunoprecipitation

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Table 1
Tumor specific CSC Markers.

Type Of Cancer	CSC markers	Reference
Pancreatic cancer	$CD133^+/CXCR4^+$, $CD24^+/CD44^+$, c-$Met^+/CD44^+$, $CD24^+/CD44^+/ESA^+$, and $ALDH1^+/CD133^+$	Reynolds and Weiss (1996)
Leukemia	$CD34^+/CD38^-$	van Rhenen et al. (2007)
Liver cancer	$CD133^+/CD45^+/CD90^+$	Grosse-Gehling et al. (2013); Herrera et al. (2006)
Malignant Melanoma	$CD20^+/CD166^+$/Nestin	Parmiani (2016)
Glioblastoma	$CD133^+/ABCG2^+$	Wee et al. (2016)
Lung cancer	$CD117^+/CD90^+$/EpCAM	Donnenberg et al. (2012)
Breast cancer	$CD24^{-/low}/CD44^+$, ALDH1	Fillmore et al. (2010); Ginestier et al. (2007)

subsequent loss of genomic stability. Cellular functions such as differentiation and viability of cellular function, are characteristics noted in most of malignant process (Brown and Robertson, 2007; Jackson-Grusby et al., 2001). The possible explanation proposed for this finding is that there is hypo-methylation of tumor suppressor genes. Further, a chromatin immune-precipitation (CHIP) assay was performed with Histone-3 lysine 27 (H3K27me23) or H3 antibodies (Bestor and Ingram, 1983; Chedin, 2011). The result showed suppression of Enhancer Zeste Homologue 2 (EZH2)-regulated target genes in DNMT1, indicating that the Polycomb gene (PcG) complex contributes to DNA methylation by regulating leukemia stem cells (Bestor and Ingram, 1983; Chedin, 2011; Trowbridge et al., 2012). PcG is an epigenetic modifier that is involved in the encoding of epigenetic silencers (Ringrose, 2007).

3. Wnt signaling pathway and CSC epigenetics

The Wnt signaling pathway is a crucial regulator of various cell activities including differentiation, proliferation, self-renewal, and cell movement (Mohinta et al., 2007). Research shows that any aberration in this pathway can lead to tumor progression (Aguilera et al., 2007; Howe and Brown, 2004). Wnts consist of 19 glycoproteins that can bind to cell surface receptors with different combinations (Cadigan and Nusse, 1997; Katoh, 2002). In canonical pathways, the Frizzled transmembrane receptor and low-density lipoprotein receptor-related protein (LRP5/6) transduce Wnt signaling by stimulating β-catenin, a critical protein that regulates growth-promoting genes (Cadigan and Nusse, 1997; Mohinta et al., 2007). The pathway is co-regulated by various positive and negative regulators; loss of negative regulators thereby leads to upregulation of gene transcription and results in tumor development (Bafico et al., 2004; Klopocki et al., 2004; Ugolini et al., 1999; Veeck et al., 2006).

Wnt binding to Frizzled causes aggregation of Dishevelled (Dvl) to form a scaffold-like structure at the plasma membrane (Bilic et al., 2007). This structure stimulates clusters of LRP5/6 that are phosphorylated by casein kinase I, followed by association of Axin, glycogen synthase kinase 3-β (GSK-3-β), and adenomatous polyposis coli to LRP singlosomes (Bilic et al., 2007). This inhibits the activity of GSK-3-β and accumulation of β-catenin in the cytoplasm (Gordon and Nusse, 2006).

Recently, methylation of the promoter of genes involved in the Wnt signaling pathway has been identified in the case of breast cancer (Ai et al., 2006; Suzuki et al., 2008; Veeck et al., 2006; Virmani et al., 2001). Wnt inhibitory factor-1 (WIF1) is a Wnt-binding protein that acts as a tumor suppressor and is also found to be downregulated in many tumors such as prostate, lung, and melanoma (Virmani et al., 2001). Methylation-specific PCR revealed hypermethylation of WIF1 in 16 of 24 tumor tissue samples (Ai et al., 2006). Furthermore, DNMT1 and DNMT3-β work in a synchronised fashion to cause methylation of WIF1 (Ai et al., 2006).

The secreted frizzled-related protein (sFRP 1–5) family acts as a Wnt antagonist. SFRP1 is mostly silent in many tumor types (Shulewitz et al., 2006). Real-time PCR analysis shows that sFRP1, 2, and 5 are methylated and downregulated in breast cancer cell lines (Suzuki et al., 2008).

Various agents are being tested as Wnt pathway inhibitors, such as nonsteroidal anti-inflammatory drugs and thiazolidinedione (an antidiabetic agent), which have already been approved for therapeutic use and are under clinical trials (Barker and Clevers, 2006; Lee, H.J. et al., 2009; Smith et al., 2000). Two monoclonal antibodies (Wnt ligand neutralizers and Wnt receptor Fz and LRP inhibitors) that target Wnt signaling are also in clinical trials (Le et al., 2015).

3.1. Epigenetics in relation to sFRP4

It has been observed that WNT/β-catenin/GSK-3 signaling is important for proliferation and self-renewal of normal and CSCs due to its multiple genetic alterations in association with human tumorigenesis, which has been shown in medulloblastoma, hepatocellular cancer, and leukemia (Karim et al., 2004).

In previous studies, the sFRP family has shown the ability to chemosensitize CSCs derived from various tumor cells to treatment (Deshmukh et al., 2017), resulting in a decreased proliferation rate and increased induction of apoptosis, particularly during tumor development when the cells are hypermethylated and inducing transcriptional silencing (Suzuki et al., 2002, 2004). In cancers such as glioblastoma multiforme, the down-regulation and gene-silencing of the sFRP family through epigenetic modification has been demonstrated (Schiefer et al., 2014).

SFRP4 is a Wnt antagonist (Warrier et al., 2013). In a recent study, sFRP4 was used as a biomarker for colorectal cancer risk (Lao and Grady, 2011). It has also been reported that epigenetic events cause methylation of CpG islands, resulting in the down-regulation of sFRP4 (Schiefer et al., 2014). Thus, the Wnt pathway is not obstructed, resulting in tumorigenesis.

Many studies suggest that various signaling pathways are also responsible for the maintenance and survival of CSCs (Takebe et al., 2015). Examples of such signaling pathways include – Wnt/β-catenin, Hedgehog, and Notch (Takebe et al., 2015). All of these pathways are involved in the development and growth of cells in the body under normal circumstances; however, one or more aberrations can give rise to cancer (Takebe et al., 2015).

4. Notch signaling pathways and CSC epigenetics

Like Wnt, the Notch signaling pathway is conserved during eukaryotic phylogeny and plays a role in regulating embryonic and adult stem cell fate in mammals (Nichols et al., 2007); (Piazzi et al., 2012). The Notch pathway's role in CSC survival could be related to the activation of tyrosine kinase PKB (McKenzie et al., 2006), a well-known mechanism to escape cell death and initiate chemo-resistance. Furthermore, the Notch target gene *IL6* mediates drug resistance in prostate cancer cells (Wegiel et al., 2008). The epigenetic regulation of genes could be involved in various CSC pathways, such as p300 mediating the activation of NF-$_K$B by signal transducer and activation of transcription 3 (STAT3) (Lee, H. et al., 2009). Activation of NF-$_K$B leads to stimulation of IL6 production, which in turn activates STAT3. IL6 plays an important role in CSC chemo-resistance and self-renewal

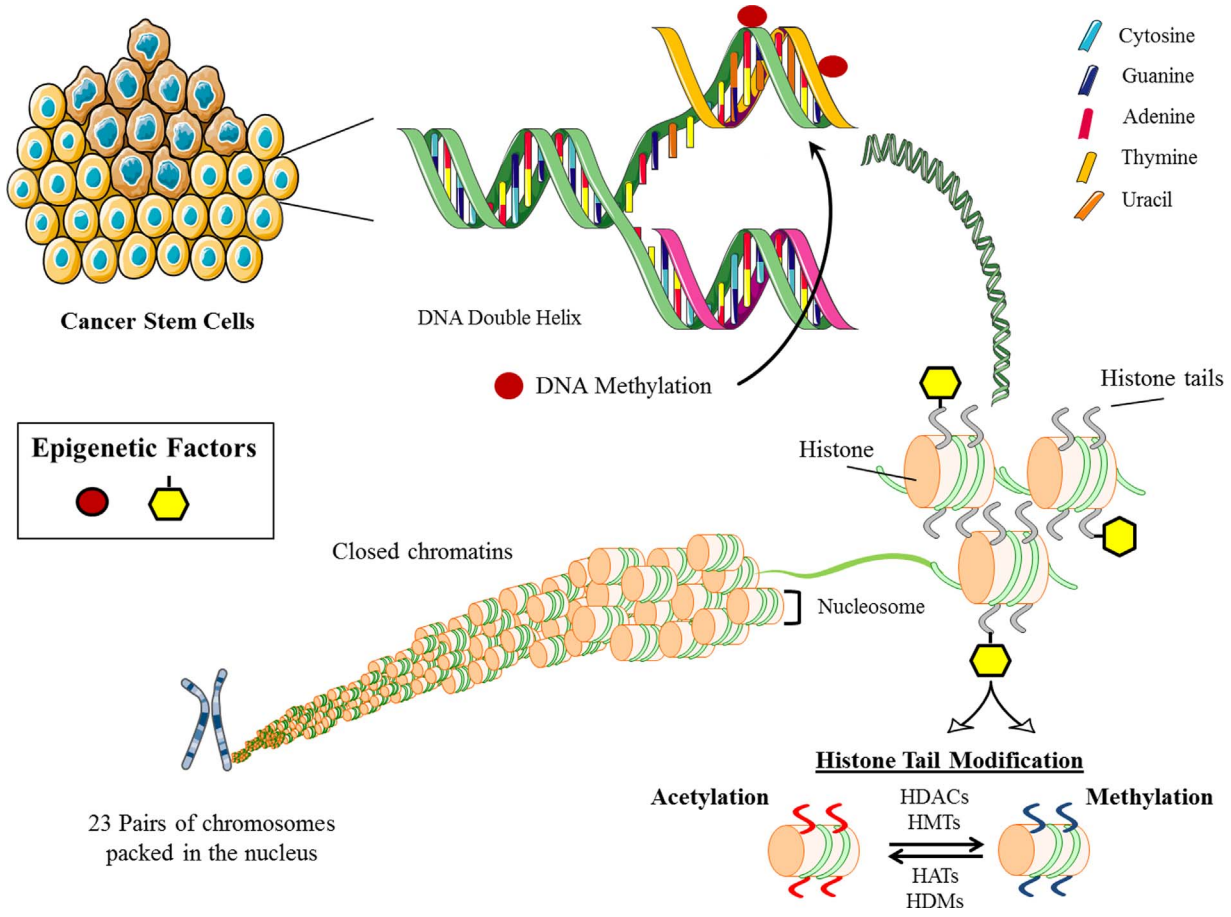

Fig. 1. In DNA methylation, methyl marks are added to DNA bases to repress gene activity, influencing the way genes are expressed without changing the underlying DNA sequence. Furthermore, other epigenetic factors bind to histones, modifying their tail. Histone tail modification controls when the chromatin complexes open up and allow their DNA to be read. (HAT: Histone acetyltransferase; HDAC: Histone deacetylase; HMT: Histone methyltransferases; HDM: Histone Demethylases).

capacity (Sansone et al., 2007). Also, Inhibition of the Notch signaling pathway depletes pancreatic progenitor cells, which exhibit similar phenotypic characteristics to CSCs (Apelqvist et al., 1999; Li et al., 2007). However, induced Notch activation prevented epithelial differentiation, resulting in increased maintenance of undifferentiated pancreatic progenitor cells (Hald et al., 2003). The Notch pathway also helps in maintaining the population of CSCs in the tumor microenvironment. Thus, this pathway can be a therapeutic target for cancer treatment (Abel et al., 2014).

Deregulation of Notch signaling has been found to be common to many tumors (Piazzi et al., 2012). It involves communication between contiguous cells via transmembrane ligands and receptors (Gomez-del Arco et al., 2010; Murthy et al., 2012). This inter-cellular communication triggers proteolytic cleavage of the receptor, which in turn releases an intracellular fragment that regulates target gene expression (Gu et al., 2012). The pathway involves five types of ligands – Delta-like ligand (DDL) 1, 3, and 4, and Jagged1 and Jagged2, as well as four receptors (Notch 1–4) (Gu et al., 2012). A recent study suggests that DLL1 is regulated by epigenetic processes in gastric cancer (Piazzi et al., 2012). In this study, the promoter of DLL1 was found to be unmethylated in a mouse model of gastrointestinal cancer (Piazzi et al., 2012). It was reported that hypermethylation of the DLL1 promoter leads to its silencing and repression of Notch signaling in gastric cancer (Piazzi et al., 2012). The data linking the Notch signaling pathway and epigenetic deregulation in CSCs are not well established, though we could elucidate the role of Hes proteins, which could activate or inhibit sets of genes by recruiting histone acetylates and deacetylase (Fischer and Gessler, 2007). A deeper dissection of the Notch-Hes pathway would enable the further role of Hes targets, such as p53 inhibitor MDM2 and Gata transcription factors, to be unveiled.

5. Hedgehog signaling pathway and CSC epigenetics

The Hedgehog (Hh) signaling pathway is mainly involved in tissue repair, embryonic development, and epithelial-to-mesenchymal transition of cells (Beachy et al., 2010). Hh ligands involved in signaling – Sonic Hedgehog (Shh), Indian Hedgehog (Ihh), and Desert Hedgehog (Dhh), undergo cleavage and produce a signaling protein with dual lipid modifications (Chen et al., 2011; Perler, 1998). In mammals, the three mechanisms that can cause mutation in the Hh pathway include ligand-independent and ligand-dependent, excessive expression of Hh ligands, and the generation of a CSC phenotype (Teglund and Toftgard, 2010). Hh molecules are paracrine molecules that bind to patched (PTCH) receptors, which in turn activate smoothened (SMOH) and translocate glioma associated homolog (GLI)-1, 2, and 3 to the nucleus to modulate gene expression. This signal transduction pathway is required for self-renewal and proliferation of cerebellar, retinal, and pancreatic CSCs (Jiang and Hui, 2008). Furthermore, GLI transcription activates *BMI1*. The Hh-BMI1 axis plays an important role in medulloblastoma progression (Liu et al., 2006). *BMI1* down-regulation leads to reduced protein kinase B (PKB) phosphorylation, impairing cell survival after chemotherapy (Qin et al., 2008). Thus, inappropriate signaling or defects in the Hh pathway can lead to cancer (Teglund and Toftgard, 2010). Active Hh signaling can interfere with cancer treatment by inducing an aggressive or resistant tumor response to treatment (Teglund and Toftgard, 2010). DNA methylation also plays an important role in regulating the Hh pathway. For example, hypomethylation of the Shh promoter causes over-expression of Shh ligands

in breast as well as gastric cancer (Cui et al., 2010). This over-expression is due to the binding of NF-$_k$B, which initiates transcription of Shh (Duan et al., 2015). Thus, Hh signaling is upregulated in breast cancer cells, enabling them to continuously self-renew (Duan et al., 2015). Interestingly, *EZH2* is upregulated in prostate cancer, predicting recurrence and simultaneously activating the polycomb repressive complex (PRC1 and PRC2), which is required for progression of cancer (Vormittag et al., 2009). Moreover, EZH2 can silence the p27 tumor suppressor gene through histone H3 lysine 27 methylation, facilitating pancreatic stem cell survival (Ougolkov et al., 2008).

In summary, the clinical contribution of *EZH2* and other PcG genes in epigenetic modulation of CSCs is largely unknown, though *BMI1*'s role in promoting resistance to therapy has made progress. However, *BMI1*'s function could vary from one tissue to another (Pietersen et al., 2008).

6. DNA methylation

The process of methylation occurs with the help of the enzyme DNA methyltransferase (DNMT) at the cytosine base that is generally adjacent to the guanine base of DNA, converting it to 5-methylcytosine (5-mC). This results in two methylated cytosine residues closer to each other on different strands of DNA (Fig. 1). (Asselin-Labat et al., 2010; Brown and Robertson, 2007; Robertson, 2005). DNMTs also provide maintenance to CSCs by constantly replenishing epithelial cells, but the molecular pathway responsible is still unclear. It has been discovered that the expression of DNMT1 is much higher in mammary tumors (Pathania et al., 2015)

6.1. Demethylation in CSCs

The depletion of DNA methylation in genes is a very common characteristic of many types of cancers (Swami, 2010). One of the reasons for hypomethylation can be mutation in DNA methyltransferases, and its association with cancer has only been recently reported (Swami, 2010).

The process of removal of the methyl group from DNA is known as demethylation. It is an important process to overcome gene silencing or tumor progression. There are two ways by which DNA can be demethylated – passive and active. Passive DNA demethylation occurs in newly synthesized DNA with the help of DNMT1, whereas active demethylation occurs in DNA by sequential modification of cytosine bases. It was also found that the addition of the demethylating agent 5-aza-2′-deoxycytidine can reverse the development of prostate CSCs by decreasing their stemness properties (Tian et al., 2012).

6.2. Hypermethylation in CSCs

Hypermethylation can occur in the promoter of tumor suppressor genes and lead to cancer. This process is being studied to use it as a treatment modality by reversing hypermethylation and ultimately terminate cancer. Hypermethylation of the p*53* gene is necessary for the regulation of apoptosis in the cell (Javeri et al., 2013; Lassacher et al., 2008; Shamsara et al., 2009).

In a healthy cell, apoptosis is triggered by any kind of change in the normal cell cycle or metabolic processes such as alteration in growth rate and intercellular signaling and exposure to radiation or toxic substances. The p53 gene is a well-known apoptosis regulatory gene (Levine, 1997). In a normal cell, *p53* is hypermethylated; however, a mutation can lead to deamination of 5-methyl cytosine, leading to the inactivation of *p53* and inhibition of apoptosis (Rideout et al., 1990). A nuclear protein called mouse double minute 2 (MDM2) is known to repress the function of *p53*. However, MDM2 is obstructed by cyclin-dependent kinase inhibitor 4a gene (INK4a), which encodes for 2 proteins: p16INK4a and p14ARF. p14ARF is involved in suppression of MDM2, therefore allowing p53 to carry out apoptosis of the cell. Due to mutation, hypermethylation of INK4a/ARF leads to inactivation of p14ARF, and thus *p53* is inhibited by MDM2, which causes abnormal behavior in the cell (Yin et al., 2002). The two main pathways responsible for apoptosis in a cell under normal conditions are mitochondrial and caspase-mediated pathways. The mitochondrial pathway mainly causes apoptosis by providing hypoxic conditions to the cells with the help of apoptotic linked proteins belonging to the BCL-2 family. B-cell lymphoma-2 (BCL-2)/adenovirus E1B interacting protein (BNIP3) is activated and localized to the mitochondria, resulting in hypoxic conditions for the cell (Regula et al., 2002). However, the CpG islands in the promoter region of BNIP3 are susceptible to hypermethylation, which can help the cancer cell escape apoptotic execution (Murai et al., 2005).

6.3. Hypomethylation in CSCs

Hypomethylation is almost always associated with hypermethylation in a genome but at a different sequence (Ehrlich, 2009); however, it is less frequent than hypermethylation. Hypomethylation is said to increase the chances of mutation in a gene, and hence the probability of carcinogenesis due to hypomethylation is much higher (Baker et al., 2010). It is also involved in chromosomal instability that can either arise due to chromatin rearrangement or any other mutation leading to cancer (Ferguson et al., 2015). It has been studied vastly and is much more understood in relation to its role in carcinogenesis as compared to other processes.

The percentage of methylation throughout the chromosome is unevenly distributed, with CpG islands mainly found in the unmethylated region of the chromosome (Rollins et al., 2006). In healthy tissue, around 3.5–4% of the cytosine has been found to be methylated under normal conditions (Ehrlich et al., 1982; Ehrlich et al., 2002). Mostly, the satellite and interspersed repeat sequences of the chromosome are methylated; however, some unidentified regions of the DNA in human brain are also found to be methylated (Rollins et al., 2006). Disruption or alteration of epigenetic regulation can cause hypermethylation at specific regions and hypomethylation of other sequences in the chromosome (Handy et al., 2011). Hypomethylation can occur in the methylated regions including the promoter of CpG islands, inactivated X chromosome, any repeated sequences, and intragenic sequences in the human chromosome (Jin et al., 2011).

It has been demonstrated that the frequency of hypomethylation in the genomes of cancer cells is very high compared to a healthy genome by determination of the levels of 5-methyl cytosine (Gama-Sosa et al., 1983; Goelz et al., 1985). Studies undertaken in the past 20 years that focused on DNA methylation have reported that the progression of cancer is greatly dominated by hypomethylation along with multiple hypermethylation of CpG islands (Ehrlich, 2002; Esteller and Herman, 2002; Fruhwald and Plass, 2002).

The global hypomethylation rate in colon carcinoma and adenocarcinoma is about 8–10% (Goelz et al., 1985). The genes involved in colorectal carcinoma, including CDH3 (P-cadherin) and CD133, are found to be demethylated. Similarly, in colon cancer, *LINE-1* has been observed to be hypomethylated (Hibi et al., 2009). The CpG islands in the promoters (CpG Island methylator phenotype) are always found to be hypermethylated, which in turn represses further transcription of tumor suppressor genes (Greenblatt et al., 1994). *CDKN2A/p16* is also methylated in colorectal cancer (Goto et al., 2009).

7. Histone modification in CSCs

Histones are highly alkaline (basic pH) proteins that package DNA into dense units (called nucleosomes) in the nucleus of eukaryotic cells (Kouzarides, 2007). Arginine and lysine are the amino acids mostly contributing to the alkaline nature of histones (Muller and Muir, 2015). A covalent post-translational modification such as acetylation, methylation, and phosphorylation that can alter gene expression is known as

histone modification (Kouzarides, 2007). It can initiate various biological processes such as repair of damaged DNA and activation by uncoiling the chromatin, or deactivation by compacting chromatin to effect gene transcription (Bannister and Kouzarides, 2011).

Each histone is involved in a particular reaction and thus the quantitative analysis of histones can help in understanding the role of epigenetics in cancer (Fig. 1).

Histone H3 at lysine 27 has been found associated with adult stem cell and cancer cell differentiation (Sakaki et al., 2015). However, it is still not clear whether the histone H3K27me3 contributes to CSCs in any way (Sakaki et al., 2015). Thus, a study was performed on the ovarian CSC line – A2780, to detect the impact of H3K27me3 in CSCs with the use of an H3K27 demethylase inhibitor – GSKJ4, which concluded that it might have an inhibitory impact on CSCs (Sakaki et al., 2015).

The main co-factors that are involved in histone modification and epigenetic changes are histone acetyltransferases (HAT), histone methyltransferases (HMT), and histone deacetylaces (HDAC) (Kouzarides, 2007). Histone acetylation is responsible for uncoiling of DNA (Fig. 1), accelerating transcription, and hindering DNA methylation; whereas, histone deacetylation leads to gene silencing (Eberharter et al., 2005; Kim et al., 2009). Thus, histone modification controls various epigenetic processes and is suspected to be involved in tumor progression (Zentner and Henikoff, 2013). Studies have shown that histone H3-K9 and DNA methylation regulate telomerase reverse transcriptase (hTET) expression that controls the binding of c-myc at the E-box 1 site of the hTET promoter (Iliopoulos et al., 2009).

7.1. Histone acetylation

Acetylation occurs at many levels in the nucleus, such as transcription activation, DNA repair, and cell cycle regulation. Histone acetyltransferase is mainly involved in transcription since the addition of an acetyl group causes denaturation in DNA, which can then initiate transcription. The process of acetylation in cells can be reversed with HDACs. Recently, it has been shown that HDAC inhibitors have the capability to induce differentiated cancer cells to gain stem-like properties and enter a quiescent state (Debeb et al., 2016).

Similarly, H4K20 (H4K20me3) also plays an important role in altering the DNA methylation status. It has been shown that the loss of trimethylation of H4K20 and acetylation of H4K16 (H4K16Ac) is associated with DNA hypomethylation in various tumors (Fraga et al., 2005). Histone modifications are also associated with increased recurrence risk in prostate cancer (Seligson et al., 2005).

7.2. Histone methylation

Gene silencing with the help of polycomb repressive complex 2 (PRC2), DNA methylation or the combination of both in CSCs suppresses their differentiation (Momparler and Cote, 2015). PRC2 consists of enhancer of zeste homolog 2 (EZH2) that acts as a catalyst in the process of trimethylation of histone 3 lysine 27 (Momparler and Cote, 2015). The PRCs interact with DNMTs to silence the tumor suppressor genes during cancer progression. In CSCs, the chromatins trigger the recruitment of DNMTs, leading to *ex novo* methylation and gene silencing. However, Histone H3 lysine 27 silences tumor suppressor genes in a DNMT-independent manner (Kondo et al., 2008), showing the PcG targets extend beyond histone methylation. PcG target genes are more likely to be DNA hyper-methylated in cancers, as shown in colon and embryonic carcinoma cell lines (Ohm et al., 2007; Schlesinger et al., 2007; Widschwendter et al., 2007), suggesting that EZH2 recruits DNA methyltransferase to the promotor region of PcG target genes and results in silencing of PcG targets and tumor suppressor genes (Vire et al., 2006). This indicates the cross-talk between DNA methylation and histone, resulting in a repressive effect. Furthermore, the combined epigenetic repressive effect was established in response to cancer cell DNA damage generated by reactive oxygen species (ROS) (O'Hagan et al., 2011). Knowing this, it could be inferred that aberrant EZH2 activity, chromatin signatures, and ROS lock the adult somatic cells into an aberrant CSC state (Lund et al., 2014).

Given the importance of EZH2's role in increasing self-renewal in some hematological malignancies, therapies targeting EZH2 inhibition may promote CSC population inhibition (Kamminga et al., 2006; Momparler and Cote, 2015).

8. Role of chromatin remodeling in carcinogenesis

Recent studies show that genetic variations such as mutations, translocation or viral integration can cause inadequacy in epigenetic processes (Burrell et al., 2013). This can also be explained by Knudson's 'two-hit' hypothesis to cause malignancy in some cases (Mastrangelo et al., 2009). We know that processes such as molecular signaling or down-regulation and up-regulation of genes are involved in cancer (Martin, 2003). Thus, studying epigenetics can help in finding the main cause of cancer and eliminate it in the future.

It has been proven that various types of mutations that can cause inactivation of *Dnmt1* are involved in the process of carcinogenesis in humans. In a recent study, somatic mutation in *Dnmt1* has been shown to be related to colorectal cancer; however, the main cause of cancer was still unclear (Kanai et al., 2003). Chromatin remodeling can also alter cancer progression by inhibiting the process of methylation in genes (Sharma et al., 2010).

9. Conclusions

Based on current evidence, we suggest models integrating signaling pathways (Wnt, Notch, and Hedgehog), epigenetic factors (DNA methylation and Histone modification), and chemo-resistance mechanisms (Deshmukh et al., 2017). The understanding of cancer states and epigenetic abnormalities may play a key role in determining patterns in different CSC populations. We discussed the concept of molecular progression for epigenetic abnormalities before tumor initiation, which eventually modulates the epigenetic changes in CSCs. The role of epigenetics in CSC biology is very important considering that CSC plasticity is tightly linked to the peculiar epigenetic profiles, and PcG genes (BMI1 and Ezh2) are well established mediators of CSC epigenetic gene regulation.

Epigenetic alterations play a vital role in maintenance of stemness within the CSCs, enhancing the immortality of tumors. As different strategies have been employed to target bulk tumors, inhibitors of epigenetic modulatory enzymes such as DNMTs and histone deactylase (HDACs), are potential targets for CSC inhibition. Already, older drugs targeting epigenetics, such as various demethylating agents and HDAC inhibitors are showing promise to sensitize tumors to other therapies (Juergens et al., 2011). The tumor suppressor proteins play an important role in chromatin remodeling; however, restoration of normal chromatin remodeling by gene therapy is a potential therapeutic strategy to treat cancers with aberrant chromatin remodeling. We are convinced that epigenetic factors such as DNA and histone methylations/acetylation and miRNAs are implicated in CSC epigenetic mechanisms. However, epigenetic therapy has the potential to lead epigenetic drugs to reverse and/or delay CSC resistance and can complement existing cancer therapies. Finally, epigenetic factors related to CSCs should be considered as therapeutic targets to prevent and eradicate cancer. Future studies are required to identify PcG targets in CSCs and to link these pathways to CSCs chemo-resistance and epigenetic modulation.

Declaration of interests

The authors declare that they have no competing interests.

Author contributions

AbhiD drafted the outline and generated the figure. AbhiD wrote the manuscript. MB contributed to the manuscript. AD conceived of the study and AD, FA, and PN critically reviewed, revised, and approved the final manuscript.

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REVIEW Open Access

Cancer stem cell metabolism: a potential target for cancer therapy

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Abstract

Cancer Stem cells (CSCs) are a unipotent cell population present within the tumour cell mass. CSCs are known to be highly chemo-resistant, and in recent years, they have gained intense interest as key tumour initiating cells that may also play an integral role in tumour recurrence following chemotherapy. Cancer cells have the ability to alter their metabolism in order to fulfil bio-energetic and biosynthetic requirements. They are largely dependent on aerobic glycolysis for their energy production and also are associated with increased fatty acid synthesis and increased rates of glutamine utilisation. Emerging evidence has shown that therapeutic resistance to cancer treatment may arise due to dysregulation in glucose metabolism, fatty acid synthesis, and glutaminolysis. To propagate their lethal effects and maintain survival, tumour cells alter their metabolic requirements to ensure optimal nutrient use for their survival, evasion from host immune attack, and proliferation. It is now evident that cancer cells metabolise glutamine to grow rapidly because it provides the metabolic stimulus for required energy and precursors for synthesis of proteins, lipids, and nucleic acids. It can also regulate the activities of some of the signalling pathways that control the proliferation of cancer cells.

This review describes the key metabolic pathways required by CSCs to maintain a survival advantage and highlights how a combined approach of targeting cellular metabolism in conjunction with the use of chemotherapeutic drugs may provide a promising strategy to overcome therapeutic resistance and therefore aid in cancer therapy.

Keywords: Cancer stem cells, Metabolism, Glycolysis, Glutaminolysis, Cancer therapy, Chemo-resistance, Tumour microenvironment, Wnt signalling

Background

Chemotherapy, along with radiotherapy and hormone therapy, is the most common treatment for cancer. Due to the side effects of treatment and chemo-resistance of the tumour cells, researchers have shifted their focus to more site-specific treatments in order to achieve better results [1].

Over the past decade, a critical role of a small subset of tumour cells, known as cancer stem cells (CSCs), was established in tumour relapse and propagation [2, 3]. Most solid tumours, including breast, brain, prostate, ovary, mesothelioma, and colon cancer contain this small subset of self-renewing tumour initiating cells [4]. Conventional anti-cancer therapies inhibit/kill the bulk of the heterogeneous tumour mass, resulting in tumour shrinkage. However, it has been suggested that later, the CSCs differentiate into tumour cells and are responsible for tumour relapse (Fig. 1). The identification of novel therapies to target CSCs has been the goal of many cancer research laboratories, and recent studies suggest the CSCs undergo metabolic alterations that include low mitochondrial respiration and high glycolytic activity. Exploiting the CSCs' metabolic alterations may provide new effective therapies and diminish the risk of recurrence and metastasis [5, 6].

Tumour cell metabolism

To induce their lethal effects and maintain survival, tumour cells alter their metabolism to ensure survival, evade host immune attack, and proliferate [7]. This clever strategy of tumour cells was exposed by Otto Warburg in the 1920s when he proved that, in spite of the presence of abundant oxygen, tumour cells metabolise glucose via glycolysis to produce lactate. They adopt

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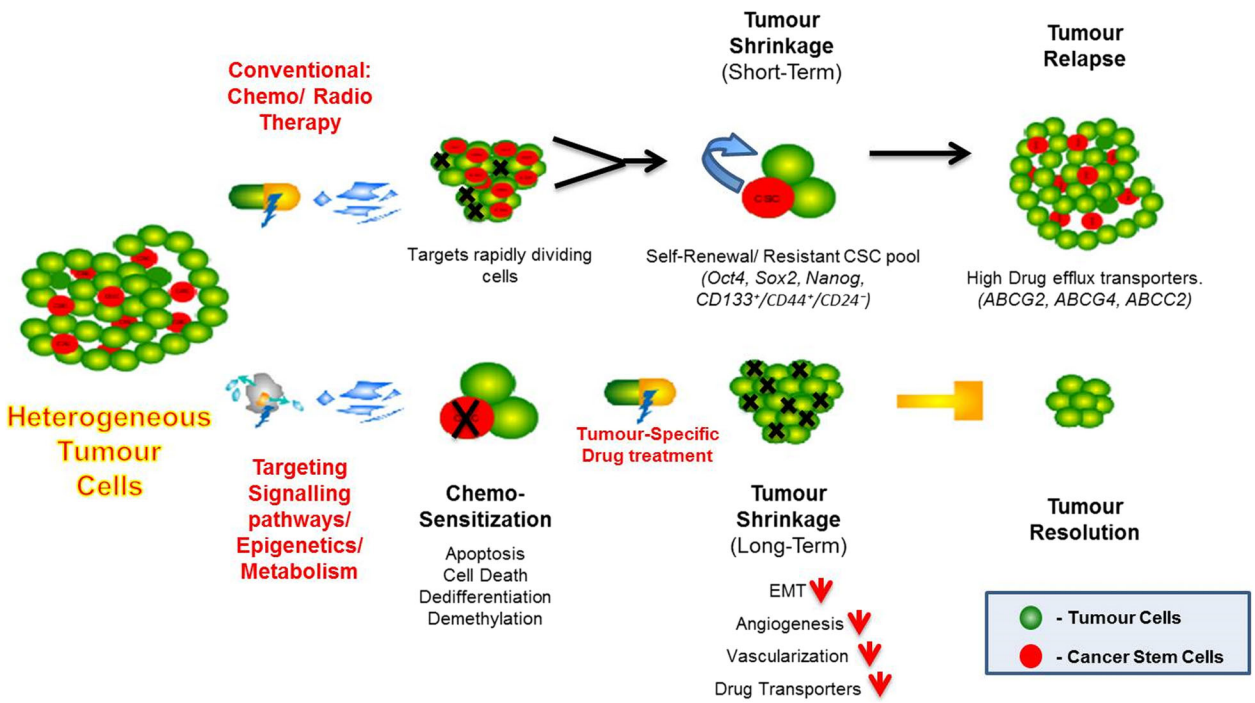

Fig. 1 Cancer stem cells (CSCs) are a small sub-population of tumour cells that are highly chemo-resistant and play a prominent role in tumour relapse. The chemo-resistant property of CSCs is believed to contribute to poor prognosis of conventional tumour treatments. Therefore, we rationalise that therapy targeting CSCs would enable chemo-sensitization by affecting downstream cellular signalling pathways of tumour cells and enable the drugs to destroy the tumour bulk.

this pathway in order to produce ATP through a fermentation process that is much faster compared to the conventional oxidative phosphorylation (respiration), and also avoids the requirement for mitochondrial oxidative phosphorylation. This meets the requirement for the tricarboxylic acid (TCA) cycle activity to be directed towards biosynthesis rather than ATP production. Inner regions of tumours are known to be hypoxic [7–10]. However, the application of anaerobic glycolysis for energy supply is just one part of the metabolic transformation of tumour cells. In order to multiply and survive, the cell must be able to replicate its genome, protein and lipid content, and other important constituents, and also pass on important biomolecules to daughter cells. To accomplish this, the tumour cells enhance the expression of glucose transporters (GLUTs) and monocarboxylate transporters (lactate/pyruvate) to ensure that glucose is delivered and that lactate is transported out of the cell [7, 11] (Fig. 2). Glutamine (via glutamate) and some of the pyruvate enters the TCA cycle to initiate the precursor supply towards biosynthetic reactions. The theoretical significance of the Warburg effect can be illustrated by the glucose uptake and solvent capacity of the cell cytoplasm, i.e. the maximum number of macromolecules that can be accommodated in the intracellular space. Thus, when the glucose uptake rate is low, glucose uptake capacity is the limiting factor and mitochondrial respiration becomes the preferred source for ATP generation. At a high glucose uptake rate, the cell identifies the solvent capacity as its prime source for generating ATP, which in turn activates aerobic glycolysis and lessens mitochondrial respiration (Fig. 2). Hence, the Warburg effect is the amicable catabolic choice for proliferating tumour cells [12]. The other interesting outcome elicited by the Warburg effect is the creation of a tumour environment that facilitates survival and proliferation of the tumours. In the process of their expansion, the tumours stretch the diffusion limits of their oxygenated blood supply and thus induce hypoxia and stabilize the transcription factor HIF. HIF triggers angiogenesis by regulating various associated factors, especially vascular endothelial growth factor [13, 14]. The other strategy adopted by these tumour cells to maximize their survival and proliferation is to increase their glutamine use for supply of biosynthetic precursors. Glutamine acts as a source of carbon and nitrogen for biosynthetic reactions of cancer cells. It gets converted to glutamate, enters into the TCA cycle, and acts as a precursor for the synthesis of important intermediates such as NADPH, anti-oxidants and amino acids such as α-ketoglutarate, aspartate, glutathione, and nucleic acids. The glutamine is converted to glutamate by

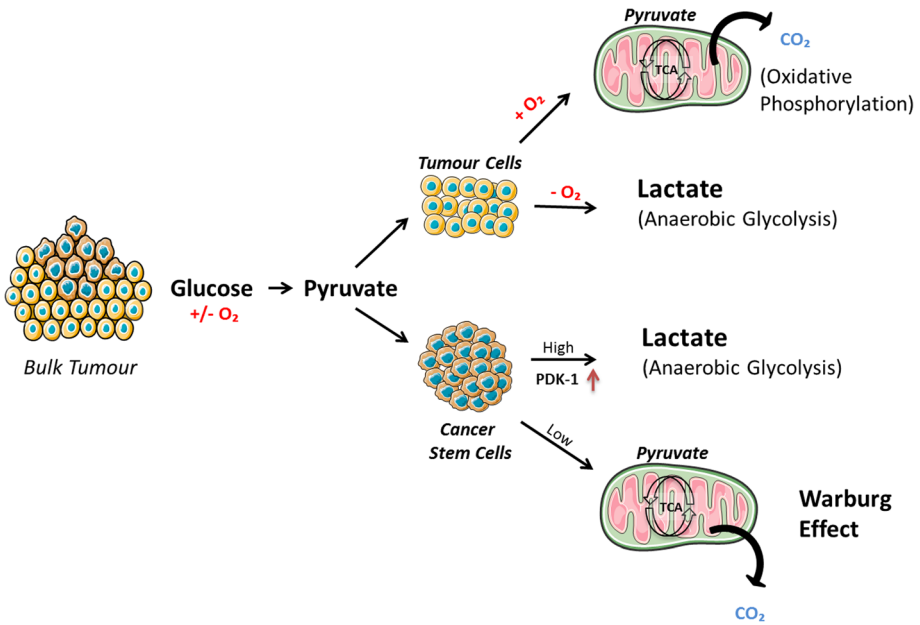

Fig. 2 Impact of glucose utilisation by CSCs and non CSCs highlights the difference in their metabolic profiles. Pyruvate enters the TCA cycle to initiate the precursor or supply towards biosynthetic reactions. The Warburg effect in turn activates aerobic glycolysis and lessens mitochondrial respiration, suggesting a preferred choice for proliferation.

the mitochondrial enzyme glutaminase. Glutaminase is highly expressed in rapidly growing tumour cells. Another link between oncogenic activation and tumour cell metabolism was determined when a study established that *c-Myc* increased glutaminase expression by suppressing miR-23a/b [7, 15, 16]. Glutamine may be partially or fully oxidised by tumour cells [17]. It acts as an energy source through catabolism or as a building block via anabolism in the body.

Cancer stem cells

The origin of CSCs is still unclear and further studies are required in each type of cancer. CSCs are known to remain in G0 phase [18, 19], the resting phase of the cell cycle, and express high drug efflux transport systems. CSCs, being in a dormant state, make it difficult for most anti-cancer drugs that target only proliferative tumour cells. CSCs exhibit specific characteristics such as self-renewal and heterogeneous differentiation capacity, small population (0.001–0.1 %), resistance to chemo/radiotherapy, high metastatic ability, sphere forming ability, and high ABC transporter expression [20, 21]. CSCs are also known to have a high migratory capacity [22], enabling their spread from the primary tumour to secondary sites [23, 24]. Various techniques have been established to isolate CSCs from the tumour mass and characterise them. CSCs are niche forming cells enriched with growth factors, and growing them in serum-free conditions containing growth factors, such as epidermal growth factor (EGF) and basic fibroblast growth factor (bFGF), maintains the undifferentiated stem cell state and induces the proliferation of self-renewing, unipotent CSCs from parental cell lines [4, 25, 26]. CSCs are characterised by specific surface markers such as $CD133^{+}/CXCR4^{+}$, $CD24^{+}/CD44^{+}$, $CD24^{+}/CD44^{+}/ESA^{+}$, $c\text{-}Met^{+}/CD44^{+}$, and $ALDH1^{+}/CD133^{+}$ in pancreatic cancer [27, 28]; $CD24^{-/low}/CD44^{+}$ in breast cancer; $CD44^{+}$ in colon/ gastric/ head and neck/ovarian cancer; $CD34^{+}/CD38^{-}$ in leukaemia cells; CD13/CD45/CD90 in liver cancer; CD117/CD90/EpCAM in lung cancer; CD20/CD166/Nestin in melanoma cancer; and $CD133^{+}/ABCG2^{+}$ in Glioblastoma Multiforme [29, 30]. CSCs also express various markers such as CXCR4/ ESA and Nestin [27]. CD44 is one of the most important CSC markers for its role in promoting tumour metastasis and invasion. CD44 has the capability to bind to its primary ligand hyaluronic acid (HA), which initiates CSC attachment to the extracellular matrix and contributes to tumour cell migration [31]. ONCOFID™-S is a conjugate of HA with SN38 (7-ethyl-10-hydroxycamptothecin) and studies have demonstrated that it showed higher anti-proliferative in-vitro activity compared to free SN38 when used against colon, gastric, breast, oesophageal, lung, and ovarian cancer cells, which overexpress CD44 [32, 33]. Therefore, a CD44-targeted therapeutic approach could be utilised for better anti-tumour drug delivery.

The CSCs with $CD44^{+High}$ and $CD133^{+High}$ expression are highly radio-resistant in colon cancer, and they also have higher expression of AKT (AKT1/2) compared to $CD44^{Low}$ and $CD133^{Low}$ cells, indicating their capacity for higher DNA repair and the ability to escape cell death/

apoptosis post radiotherapy [34]. Therefore, selective targeting of these markers can be an effective way to deliver cytotoxic drugs to CSCs.

CSCs and their metabolic alterations

Although much is known regarding metabolic pathways important for tumour survival, the potential for therapeutic metabolic alteration of CSCs still remains under investigation [35, 36]. Recent studies indicate that CSCs have different metabolic properties when compared to the tumour bulk. One such study on brain tumour CSCs revealed that these cells show a low activity of mitochondrial respiration [37]. This finding triggered the need to study the effect of glucose in the microenvironment of CSCs because glucose was estimated to be critical for the CSCs. It was found that CSCs have higher glycolytic rates than other tumour cells [38]. Glucose induced the expression of specific genes in CSCs associated with glucose metabolism and the Akt pathway (*c-Myc*, *GLUT1*, *HK-1*, *HK-2*, and *PDK-1*), which contributes to the rise in the CSC population [38]. Glucose utilisation by CSCs and non CSCs was compared by measuring their glucose consumption and lactate production rates in order to establish evidence for the difference in metabolic profiles of CSCs and the bulk of the tumour. It has been observed that glucose uptake, lactate production, and ATP content are elevated in CSCs as compared to the non CSCs (Fig. 2) [39–41]. Many crucial molecules involved in glucose metabolism have been studied in relation to the metabolism of CSCs, such as hexokinase-1 (HK1), hexokinase-2 (HK2), pyruvate dehydrogenase kinase 1 (PDK1), and glyceraldehyde-3-phosphate dehydrogenase. The PDK1 levels are high in the CSC population (Fig. 2). PDK-1, via the TCA cycle, phosphorylates pyruvate dehydrogenase and suppresses the pyruvate to acetyl-CoA conversion. Furthermore, suppressing the metabolic flow of pyruvate in mitochondria induces the conversion of pyruvate to lactate in the cytosol [38, 42]. HK-1 and HK-2 both catalyse the conversion of glucose to glucose-6-phosphate in glycolysis, but the levels of HK-2 are lower in CSCs while that of HK-1 are higher, suggesting that HK-1 maintains CSCs' glycolytic activity. Interestingly, *HIF-1α* and *c-Myc* expression (affects HK-2 expression) didn't change in CSCs and tumour cells. The increase in expression of proteins in the Akt signalling pathway bestows CSCs with a longer life span [8, 38].

Palorini et al. [41] studied the effect of glycolysis inhibition and glucose deprivation on the CSC cell line 3AB-OS, which was derived from the human osteosarcoma cell line MG63. They reported that the 3AB-OS cells require glucose for survival and proliferation. The absence of glucose caused death of the CSC cell line. Glutamine deprivation led to a decline in the MG63 population, which suggested that the 3AB-OS population was not greatly affected by withdrawing glutamine.

Hence, incorporating these features into therapeutic strategies to treat cancer can produce an extensively efficient treatment for various cancers. Also, combining glycolytic inhibition strategies with existing chemotherapy can also help eliminate tumour load completely because the CSCs will also be targeted [41].

Targeting metabolic regulators

Understanding the mechanism by which CSCs are chemo-resistant and initiate tumour relapse is very important in order to address cancer therapy and to understand CSC biology (Fig. 1). B-cell lymphoma (Bcl-2) protein and its family members are known metabolic regulators, and it is recognised as a crucial mediator of mitochondrial apoptotic signalling. Its metabolic role was confirmed by the presence of Bcl-2 associated death promotor (BAD) in complex with glucokinase [43]. Glucokinase has a low affinity for glucose transporter proteins and is purely substrate driven, making it an ideal substrate sensor to detect glucose in pancreatic Beta cells and hepatocytes [43]. The activation of glucokinase is driven by phosphorylation of BAD by kinases such as Akt. The BAD`s pro-apoptotic capacity is inhibited when bound to glucokinase. However, dephosphorylated BAD, on dissociation with glucokinase, will bind to Bcl-2/xl, initiating apoptosis. Furthermore, it has been shown in some cancers that inhibition of BAD phosphorylation decreases cancer cell survival [44, 45].

The glucokinase complex and BAD accumulation will also promote glycolysis, which favours proliferation and CSC biosynthesis. However, dephosphorylation of BAD shifts the balance towards cell death and inhibits the metabolic signals necessary for high glucose flux to enable cell survival regulation [46].

The Bcl-2 protein family impairs the cell's ability to release apoptogenic protein cytochrome c from the mitochondria by mediation of the balance between cell survival and apoptosis. It achieves this by binding to the pro-apoptotic proteins Bcl-2 associated X protein (BAX) and Bcl-2 homologous antagonist killer [33, 47]. While the mechanism of Bcl-2 expression in CSC chemo/drug resistance is still unclear, and it might be due to chromosomal translocation or another pathway, it was demonstrated that leukaemia $CD34^+$ cells expressed Bcl-2 and Bcl-X [48], and Bcl-2 was highly expressed in breast $CD44^+/CD24^-/^{low}$ CSCs [49]. To further understand the role of the Bcl-2 protein family, it was demonstrated that Bcl-2 expression in $CD133^+$ human hepatocellular carcinoma cells (HCC) can be regulated by the Akt signalling pathways, the inhibitor specific for AKT1 reduced this cell survival protein expression significantly, indicating that $CD133^+$ HCC contribute to chemo-resistance

through preferential activation of AKT/PKB and Bcl-2 cell survival response [50].

One of the mechanisms for CSCs to achieve their metabolic shift is through modifications of metabolic and apoptotic roles of Bcl-2 family proteins. The metabolic alterations of this family of proteins may prove potent in increasing cancer cells susceptibility to apoptosis and affecting tumorigenic metabolic reprogramming.

Targeting drug transporters

CSCs are known to possess a high efflux system that disables the chemo-therapeutic drugs' activity, resulting in the formation of highly drug-resistant tumours [31]. CSCs have been found to express several Adenosine triphosphate–binding cassette (ABC) transporters such as ABCB1/P-gp/MDR1, ABCG2/ BCRP/MXR, and ABCB5 [51, 52]. The ABC transporters are highly dependent on ATP generation in CSCs; thus, targeting CSC metabolism/glycolysis would lead to depleted ATP production and inhibition of ABC transporters. ABCG2 is considered as a high capacity transporter of various substrates including chemotherapeutic drugs [53]. With this in mind, it is has been suggested that $ABCG2^+$ tumour cells can represent CSCs, which are known for their drug-resistance. Higher ABCG2 expression has been observed in various CSCs from lung [54], pancreas [55], and liver [56], and is co-expressed with CD133 in melanoma and pancreatic cancer cell lines [57, 58]. It is suggested that ABCG2 expression is upregulated by hypoxia via hypoxia-inducible transcription factor complex HIF-1α and HIF-2α signalling [59].

ABCB1/ P-glycoprotein (P-gp) /MDR1 are known to be expressed in the majority of drug resistant tumours. Being a product of the multidrug resistance (MDR1) gene, it acts as an ATP-dependent efflux pump to various anti-cancer drugs [60]. CSCs derived from pancreatic tumour cells have higher expression of ABCB1 and ABCG2 [61]. Furthermore, the first generation inhibitors (FGI) verapamil and PSC833 were unable to efficiently inhibit mitoxantrone efflux in leukaemic CSCs, showing that high expression of ABCB1 would lead to the development of chemo-resistant cells [62]. Second generation inhibitors (SGI) were structurally modified for more potency, low cell toxicity, and higher specificity, and include dexverapamil [63] and Valspodar (PSC833) [64]. Another SGI, PSC833, showed higher potency compared to the FGI`s, although this is also an inhibitor of cytochrome P-450 and caused drug-drug interaction associated with anti-cancer drugs [65]. Third generation inhibitors (TGI) utilise nano-molar concentrations to have more potency at reversing MDR compared to TGI and SGI. Zosuquidar (LY3359) [66], an oral P-gp inhibitor used in treating acute myeloid leukaemia, significantly increases the uptake of daunorubicin, idarubicin, and mitoxantrone [67]. Another inhibitor, Tariquidar [68], which is used at very low concentrations (25–80 nM), has a high P-gp affinity that inhibits its ATPase activity [69]. Although it has been used as a potent P-gp inhibitor in clinical trials [70], recent studies have shown that Tariquidar is both a substrate and inhibitor of P-gp, depending on its in-vivo dosage [71]. Fourth generation inhibitors (FGI) are natural compounds or plant extracts exhibiting less cytotoxicity and better oral bioavailability. In-vitro analysis showed MDR reversal of ABC drug transporters when treated with extracts of Chinese herbal plants such as flavonoids or stilbenes [72]. Some natural compounds such as trabectedin, cytarabin, and halaven, have been approved for clinical use based on their strong MDR reversal activity by impacting on ABC drug transporters [73–76].

ABCB5β (a half-transporter) has been found in malignant melanoma and breast cancer, and is known to mediate doxorubicin resistance [77]. The $ABCG5^+$ cells represent 2–20 % of the melanoma tumours and have been shown to successfully recapitulate the tumour in immuno-deficient mice; however, these tumours were unable to regenerate $ABCG5^+$ cells, suggesting their limited stemness capacity [20].

Inhibition of ABC transporters can also cause toxicity to a patient's normal stem cells, since these have an enhanced DNA repair mechanism, particularly bone marrow-derived stem cells. In addition, ABCG2 and ABCB1 play a pivotal role in maintaining the blood brain barrier, and interfering with their normal function could have drastic consequences [78].

Targeting the tumour microenvironment

Tumour progression is due to adaptive cellular responses such as dormancy, invasiveness, and chemoresistance in the tumour metabolic microenvironment [79]. Adaptive behaviour of CSCs in this heterogeneous microenvironment is one of the characteristics of CSCs [80]. The tumour microenvironment plays a pivotal role in cancer cell progression, particularly for CSCs, and it mostly involves hypoxia, nutrition, and low pH [81].

Hypoxia in the tumour microenvironment allows pro-angiogenic factors to stimulate new vessel growth within the solid tumour, although the vessels tend to be immature and exhibit poor perfusion [82]. Hypoxia, due to its spatial and temporal heterogeneity in tumours, is difficult to treat [83]. The hypoxic response within the microenvironment is regulated by Hypoxia inducible transcription factors, HIF-1α/ HIF-2α. The migration, glycolytic, angiogenic, and cell survival pathways constitute the transcription targets of HIF1α [84]. Hence, targeting HIF1α is a potential therapy for cancer treatment.

In hypoxic stress, the endoplasmic reticulum (ER) is inhibited, activating the Unfolder protein response (UPR). The UPR maintains ER homeostasis and its disruption

initiates apoptosis. Aberrant activation by the UPR in the absence of the two ER membrane proteins PERK (PKR-like ER kinase) and IRE-1 (inositol-requiring) results in increased hypoxia and reduced growth rates [85, 86]. The UPR is an important cellular response mechanism in cancer, playing a role in calcium homeostasis, redox status, and glucose deprivation within the tumour.

Another potential target within the microenvironment is mammalian target of rapamycin (mTOR). During cell stress, nutrient and energy depletion within the solid tumour, mTOR activates the signalling cascades responsible for metabolism and cell survival mechanisms [87, 88]. The anti-diabetic drug metformin has exhibited potential anti-tumour activity; it reduces blood glucose levels, thereby inhibiting gluconeogenesis, and initiates AMPK (AMP-activated protein kinase) activation [89]. AMPK regulates the mTOR activity through activation of the tuberous sclerosis protein 1 complex (TSC1/2) [90].

The microenvironment of tumours is more acidic (pH 6.5–6.9) compared to normal tissues (pH 7.2–7.5), resulting in tumours having poor vascular perfusion and increased glycolytic flux [91, 92]. Knowing that tumour invasiveness is more active in an acidic microenvironment [93, 94], manipulating the tumour microenvironment pH by orally distributed systemic buffers is an effective way to increase the extracellular pH of tumours [95, 96].

Targeting glycolytic enzymes to reduce chemo-resistance in CSCs

Most cells satisfy their energy demands through glucose catabolism, which is subject to complex regulation. To inhibit glucose catabolism through the central pathway of glycolysis, various glycolytic enzymes or transporters must be targeted such as GLUT 1 - 4 [10], hexokinase [97], pyruvate kinase M2, and lactate dehydrogenase A [98].

Cancer cells have the ability to alter their metabolism in order to fulfil bioenergetic and biosynthetic requirements. The extracellular environment can be acidified by what is known as the 'Warburg effect' (a term referring to high levels of glycolytic pathway flux, even under aerobic conditions). When HIF-1α induces the expression of carbonic anhydrases, and there is an interaction with extracellular acidification, the pH ratio between the intracellular and extracellular environment is altered [99–102].The resultant pH shift affects drug absorption within the cell. At the same time, glycolytic adenosine triphosphate (ATP) production and the transporter induced over-expression of HIF-1α contribute to a decrease in the cytoplasmic retention of anti-cancer agents [37, 103].

Targeting mitochondrial respiration

The distinct metabolic profile of CSCs has been reported in a few types of cancer, demonstrating CSCs to be more dependent on mitochondrial respiration and less on glycolysis [104]. CSCs prefer oxidative phosphorylation (OXPHOS) for energy production in lung cancer [105], glioblastoma [106], pancreatic ductal adenocarcinoma (PDAC) [104, 107], and leukemic stem cells [108]. The finding in PDAC cells and PDAC-CSCs demonstrates that unlike other highly glycolytic tumour cells, the PDAC-CSCs do not depend on lactate production to generate NAD+ for anabolic respiration to support continued glycolysis and are more dependent on mitochondrial respiration [104]. OXPHOS inhibition impacts directly to the CSCs' sphere formation capacity and tumorigenic potential, indicating extreme sensitivity to mitochondrial function inhibition [104, 107]. The CSCs' strong dependence on mitochondrial electron transport chain activity on autophagic and catabolic processes makes them more resistant towards nutrient and environmental factors [104, 107]. In normal and leukaemic stem cells, a dependence on OXPHOS for energy production demonstrates the importance of mitochondrial respiration [108–111]. These findings imply an alternative approach to target tumour relapse by targeting OXPHOS in association with oncogenic pathway inhibitors in pancreatic cancer [104].

Glutaminolysis in cancer metabolism

Cancer cells metabolise glutamine, as well as glucose, to grow rapidly because it provides the required ATP and essential biomolecules such as proteins, lipids, and nucleic acids [112]. Glutamine influences the signalling pathways required for cancer cell proliferation, survival, and metabolism through regulation of mitochondrial reactive oxygen species (ROS) production [113, 114]. Activation of the PI3-Kinase-Akt pathway results in increased production of ROS in mitochondria through metabolic pathways [115]. Glutamine is first converted to glutamate by the enzyme glutaminase, and then glutamate is converted to α-ketoglutarate (αKG) by the action of glutamate dehydrogenase (or an aminotransferase). The rapidly growing tumour cells use glutamine as a carbon source for energy production and for the replenishment of TCA cycle intermediates such as pyruvate, oxaloacetate, and αKG to make up for the constant loss of citrate, which is exported out of the mitochondria for lipid synthesis. It has been observed that glutamine withdrawal in cells with increased *c-Myc* expression led to the death of the oncogenic cells [16]. Thus, it can be confirmed that cancer cells employ glutamine to provide substrates for the TCA cycle [113, 116]. Further studies have also demonstrated that the oncogene *c-Myc* impacts glutamine metabolism, thus stimulating the glutamine transporters SLC5A1 and SLC7A1 and, as a result, promoting the expression of glutaminase 1 by suppressing the expression of miR-23A and miR-23B [39].

These data provide a concrete platform to include glutamine metabolism in cancer as an integral part of cancer therapeutic strategies. Glutamine analogues such as 6-diazo-5-oxo-L-norleucine (L-DON), acivicin, and azaserine were found to demonstrate anti-cancer activities but were not formulated into drugs due to their neuro- and gastrotoxicity [117]. However, it has been shown that inhibition of glutamine metabolism via L-DON was able to reduce cancer metastasis in a VM-M3 mouse model [39, 118]. Zhou et al. [119] performed a proteomic analysis in pancreatic ductal adenocarcinoma that revealed the role of glutamine metabolism in cancer. They found that the level of glutaminase in the cancer cells was much higher compared to the normal ductal cells. In addition, the concentration of other enzymes such as cytidine triphosphate synthase, guanine monophosphate synthetase, and asparagine synthetase, which use glutamine as substrates, was found to be elevated in pancreatic cancer. This indicates that the high utilisation rates of glutamine by cancer cells are required to satisfy their need of nitrogen and energy for uninterrupted, fast growth. Paediatric acute leukaemia has been successfully treated by L-asparaginase, which catalyses the hydrolysis of asparagine to aspartic acid. This enzyme is also capable of hydrolysing glutamine to glutamic acid and ammonia, thus reducing blood glutamine levels [39].

Histone deacetylase (HDAC) inhibitors such as phenylbutyrate have been used pharmacologically to inhibit the invasive properties of breast and prostate cancer by inducing apoptosis and depleting the blood glutamine levels [120, 121]. It is generally used to treat hyperammonemia in urea cycle disorders, but it also brings down the level of glutamine in the plasma by forming a conjugate and thus helps curb tumour growth [122]. The glutamine transporters SLC1A5 (ASCT2) and SLC1A7, which are over-expressed in various human cancers such as colon, liver, colorectal adenocarcinomas, glioblastoma multiforme, and melanoma, have been attractive targets due to their role in cell survival signalling and also being a major source of glutamine delivery [123]. IL-γ-glutamyl-p-nitroanilide has been shown to inhibit SLC1A5 (ASCT2) and cause autophagy in cancer cells [39, 116]. A chemical compound termed 968 exhibited anti-glutaminase activity, which in turn suppressed the oncogenic transformation by *c-Myc* via down regulation of miR-23, which has been seen in prostate cancer and human B-cell lymphoma [39]. Another compound, Bis-2-(5-phenylacetamido-1, 2, 4-thiadiazol-2-yl) ethyl sulphide, also exhibited inhibitory effects on glutaminase, thus repressing glutamine availability to the cancer cells [117]. Ongoing and future work would aim at presenting a more detailed picture of glutamine metabolism and its involvement in cancer, which would help develop safe and effective glutamine inhibitors.

Conclusion

The targeting of CSCs is emerging as a novel therapy to eradicate the progression of various cancers. The inefficiency of traditional anti-cancer therapies lay the stepping stone for studying the metabolism of cancer cells and the pathways controlling and regulating their growth and proliferation, and converting them into formidable treatment options. Targeting the special metabolic traits of CSCs would enable the basis for the development of new therapeutic strategies to inhibit the bulk of the tumour. Clinically, targeting the CSCs resistant towards therapy and metastasis would enable long term disease free survival for the patients.

Though, drug development for CSC metabolism is gaining wide interest, it is still controversial issue as there are studies contradicting the glycolytic phenotype of CSC and oxidative state of CSCs. On the other hand, cancer cell metabolism has emerged to be one of the most fascinating and promising areas in cancer therapy research. The current research focuses on trying to understand the metabolic demands and profile of cancer cells, and design drugs accordingly in order to add a new exciting chapter to cancer treatment. Also, drugs targeting cancer metabolism can be employed for multiple cancers, which can possess a broad spectrum of activity, and are indeed under clinical trials that will likely result in new treatment options in the future [124]. Despite the limited research on the role of metabolism in CSCs and their ability to self-renew, tumour initiation, differentiation capacity, chemo-resistance and survive therapy, targeting CSCs metabolism holds great promise in translating cancer treatments. Though, combinatorial treatments involving both standard chemotherapeutic drugs and chemo-sensitizing agents on CSCs would probably be the most efficient CSC-targeted therapy (Fig. 1).

Abbreviations

ABC: ATP binding cassette; Bcl-2: B-cell lymphoma; CSCs: Cancer stem cells; HK: Hexokinase; OXPHOS: Oxidative phosphorylation; PDAC: Pancreatic ductal adenocarcinoma; PDK1: Pyruvate dehydrogenase kinase 1; PTCH1: Patched homologue; SDF1: Stromal cell derived factor 1; TCA: Tricarboxylic acid cycle; αKG: α-ketoglutarate

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Authors' contributions

AD drafted the outline and generated the figures. AD wrote the manuscript. KD contributed to the manuscript. AD conceived of the study and AD, FA,

and PN critically reviewed, revised, and approved the final manuscript. All authors read and approved the final manuscript.

Competing interests
The authors declare that they have no competing interests.

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SCIENTIFIC REPORTS

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Secreted Frizzled-related protein 4 (sFRP4) chemo-sensitizes cancer stem cells derived from human breast, prostate, and ovary tumor cell lines

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This study investigated molecular signals essential to sustain cancer stem cells (CSCs) and assessed their activity in the presence of secreted frizzled-related protein 4 (sFRP4) alone or in combination with chemotherapeutic drugs. SFRP4 is a known Wnt antagonist, and is also pro-apoptotic and anti-angiogenic. Additionally, sFRP4 has been demonstrated to confer chemo-sensitization and improve chemotherapeutic efficacy. CSCs were isolated from breast, prostate, and ovary tumor cell lines, and characterized using tumor-specific markers such as $CD44^+/CD24^-/CD133^+$. The post-transcription data from CSCs that have undergone combinatorial treatment with sFRP4 and chemotherapeutic drugs suggest downregulation of stemness genes and upregulation of pro-apoptotic markers. The post-translational modification of CSCs demonstrated a chemo-sensitization effect of sFRP4 when used in combination with tumor-specific drugs. SFRP4 in combination with doxorubicin/cisplatin reduced the proliferative capacity of the CSC population *in vitro*. Wnt/β-catenin signaling is important for proliferation and self-renewal of CSCs in association with human tumorigenesis. The silencing of this signaling pathway by the application of sFRP4 suggests potential for improved *in vivo* chemo-responses.

Chemotherapy, along with radiotherapy and hormone therapy, is the most common treatment for cancer. Due to the side effects of treatment and chemo-resistance of tumor cells, researchers have shifted their focus to more site-specific treatments in order to achieve better patient outcomes[1]. Over the past decade, a critical role of a small subset of tumor cells, known as cancer stem cells (CSCs), was established in tumor relapse and propagation[2,3]. Most solid tumors, including breast, brain, prostate, ovary, mesothelioma, and colon cancer contain this small subset of self-renewing, tumor initiating cells[4]. Conventional anti-cancer therapies inhibit/kill the bulk of the heterogeneous tumor mass, resulting in tumor shrinkage. However, it has been suggested that later, the CSCs differentiate into tumor cells and are responsible for tumor relapse[5,6]. CSCs are characterized by their tumor forming ability and expression of high levels of ATP-binding cassette drug transporters (ABCG2), cell adhesion molecules (CD44), and anchorage independent cell survival proteins (Cyclin D1), which are collectively responsible for chemo-resistance[7–9]. In human breast, ovary, and prostate cancers, several CSC populations have been identified using cell surface markers ($CD44^+/CD133^+/CD24^{-/low}$); these CSCs have shown a high clonal, invasive, and metastatic capacity, leading to resistance to radio-therapy, chemotherapeutic drugs (doxorubicin and cisplatin), and other target-specific therapy[10–12].

CSCs possess high capacity for tumor propagation and metastasis[13–15], which causes more than 90% of cancer-related deaths. The molecular mechanism of CSCs regulating metastasis is not completely understood; however, the invasive metastatic cascade involves circulation of cancer cells through the surrounding extracellular matrix in a multistep cellular operation.

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Treatment	Surface markers	$CD44^+/CD24^-$ (%)		$CD133^+/CD44^+$ (%)				
		MDA231	MCF7	PC3	LnCap	A2780 P	A2780 ADR	A2780 CIS
Untreated		58.1	36.7	24.3	62.7	2.85	19.5	2.72
sFRP4		32.3	17.2	17.2	44.1	2.99	16.8	2.21
Dox./Cisplatin		28.5	14.9	22.1	47.5	9.29	16	1.8
sFRP4 + *Dox./Cisplatin*		**25**	**1.68**	**9.16**	**10.4**	**1.88**	**15.1**	**1.23**

Table 1. Effect of sFRP4 on CSCs characterization. Using flow cytometry, Statistical analysis of CSC markers post treatment. Data are means ± percentage from 3 independent experiments.

The development and maintenance of CSCs is controlled by several signaling pathways such as Wnt and Notch. The Wnt pathway is known to mediate the self-renewal capacity of CSCs through modulation of β-catenin/TCF transcription factors. There is evidence suggesting a Wnt signaling role in CSC maintenance (as seen in murine models and humans) of non-melanoma cutaneous tumor, where CSCs are maintained by Wnt/β-catenin signaling[16]. The interactions of Wnt proteins to the receptor complex can be inhibited by binding of the ligands to endogenous Wnt antagonists such as secreted frizzled-related proteins (sFRPs)[17]. SFRP4 is one of the prominent isoforms with the capacity to chemo-sensitize tumor cells to chemotherapeutics[18,19]. Chemo-sensitization of CSCs by sFRP4 has the potential to decrease the required chemotherapeutic load to facilitate tumor resolution.

Results

Tumor derived CSCs characterization. Spheroids obtained for CSC isolation were characterized for the expression of tumor-specific CSC markers $CD44^+$ / $CD24^{-/low}$ for breast CSCs, and $CD133^+/CD44^+$ for prostate and ovarian CSCs (Table 1), by using flow cytometry. The combinatorial treatment showed significant reduction in the CSC marker population in all cell line-derived CSCs; although in A2780 prostate CSCs, cisplatin treatment showed phenotype switching to $CD44^+$ positive cells and only reduced the $CD133^+$ population; however, this switching did not affect the inhibitory effect of combinatorial treatment (see Supplementary Figure 1). The characterized CSCs were further used for functional analysis.

SFRP4 in combination with doxorubicin/cisplatin reduces the sphere forming capacity of CSCs. The CSCs derived from breast, prostate, and ovary tumor cell lines were treated with sFRP4 (250 pg) and doxorubicin (5 μM)/cisplatin (30 μM) alone or in combination. The untreated spheroids remained intact, whereas the combinatorial treatment of sFPR4 and chemotherapeutic drugs showed disruption of spheroids post-treatment (Fig. 1), indicating sFRP4's capacity to segregate the tumor spheres and allow chemotherapeutic drugs to inhibit tumor proliferation. This was further confirmed by immunofluorescence, where spheroids were labelled with $CD44^+/CD24^{-/low}$/ABCG2/KI67 for CSCs derived from breast tumors (Fig. 2a and b), and $CD133^+/CD44^+$/ABCG2/Ki67 for prostate (Fig. 2c and d) and ovarian tumors (Fig. 2e, f, and g); except for the prostate LnCap CSCs, which were not $CD44^+$ (Fig. 2d). The combinatorial treatment showed sphere disruption and a reduction in surface receptor expression compared to sFRP4 and drugs alone, indicating the effect of sFRP4 in inhibiting the spheroids' proliferative capacity.

SFRP4 in combination with doxorubicin/cisplatin reduces CSC viability. Using an MTT assay, it was observed that the combinatorial treatment of sFRP4 and doxorubicin/cisplatin significantly inhibits the viability of CSCs ($P < 0.001$, $n = 3$) compared to sFRP4 or drugs alone. Similar patterns were observed in all the cell lines (Fig. 3). Therefore, this treatment combination was used for subsequent studies.

SFRP4 and doxorubicin/cisplatin treatment downregulates the expression of CSC stemness genes. The stemness related genes *SOX2*, *Klf4*, *Nanog*, and *Oct4* are expressed in CSCs and are associated with tumor progression. Semi-quantitative PCR analysis showed the untreated CSCs expressing all the genes, but the treatment with sFRP4 alone or in combination with doxorubicin/cisplatin downregulated the expression of *SOX2*, *Klf4*, *Nanog*, and *Oct4* in all the cell line-derived CSCs. The combinatorial treatment showed maximum reduction of gene expression, indicating that sFRP4 in combination with chemotherapeutic drugs has the capacity to reverse the stem cell-like properties of CSCs (Fig. 4).

SFRP4 mediates early apoptotic events in CSCs. The disruption of mitochondrial membrane potential was investigated by using JC-1 dye. Results from the JC-1 assay demonstrated a significant increase ($p < 0.01$) in mitochondrial depolarization after treatment with sFRP4, doxorubicin/cisplatin alone, and in combinatorial treatments compared to untreated control. In all cell line-derived CSCs, maximum depolarization was observed in combinatorial treatments, indicating early stage death and apoptotic response through sFRP4 (Fig. 5a). To further confirm the apoptotic role of sFRP4 in CSCs, we studied caspase 3 activity in CSCs derived from all cell lines, which indicated increased caspase 3 activity ($p < 0.001$) in the sFRP4 alone and combinatorial treatments in comparison to untreated cells (Fig. 5b).

SFRP4 regulates protein expression in CSCs. Following sFRP4 treatment, we investigated the post-translational modifications in CSCs for ABC transporters (ABCG2), oncogenes (*c-Myc*), anchorage independent cell survival (Cyclin D1), anti-apoptotic (Bcl-xl), and pro-apoptotic (Bax) proteins. Results demonstrated the existing chemo-resistance of CSCs (Fig. 6). ABCG2 was highly expressed in the untreated groups but decreased in the presence of sFRP4, doxorubicin/cisplatin, and combinatorial treatments, with the latter

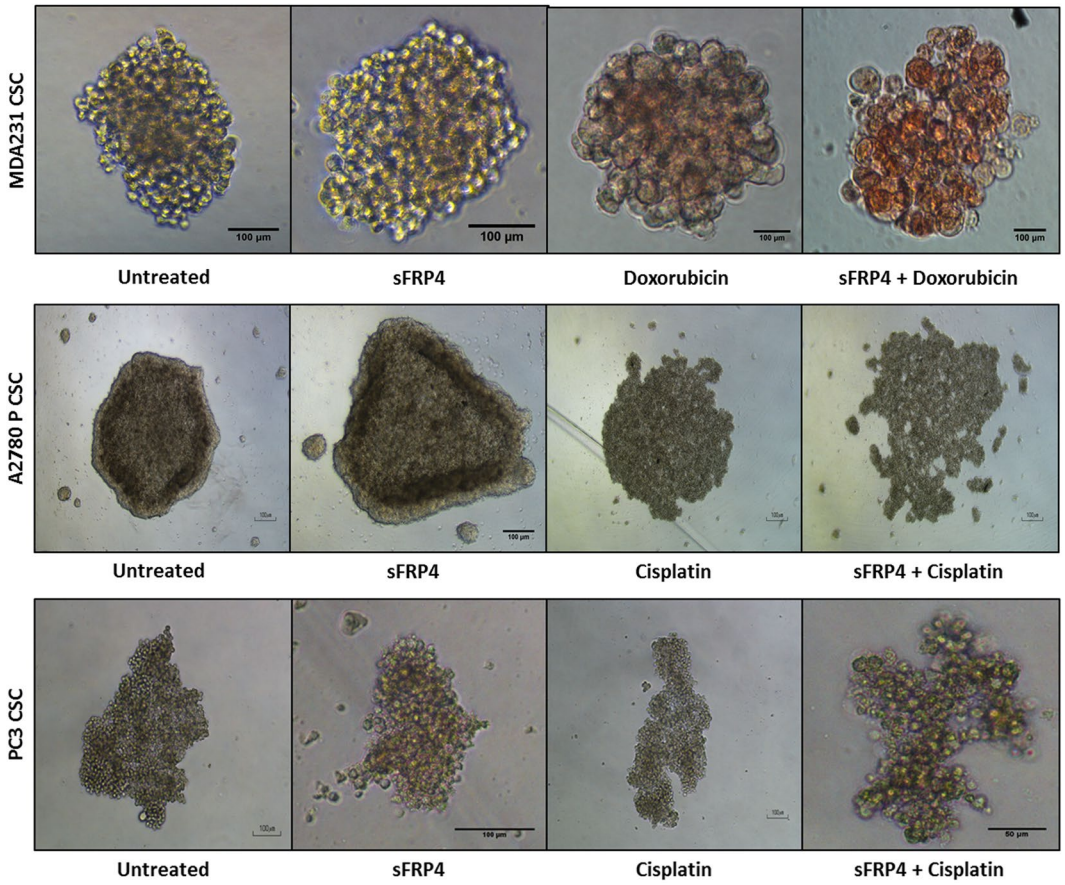

Figure 1. Effect of sFRP4 on CSC Morphology: CSCs were isolated from breast, ovary, and prostate tumor cell lines and treated with sFRP4 (250 pg) with chemotherapeutic drugs (doxorubicin 5 μM/cisplatin 30 μM). The combinatorial treatment shows the disruption of the CSC sphere. (Scale bar: 100 μm). Cells images are representative from all the experiments.

inducing the lowest expression levels. *c-Myc* had similar expression levels in all CSCs, except ovarian A2780-Cis CSCs (Fig. 6g). The levels of the proto-oncogene cyclin D1 decreased in all the combinatorial treatments of CSCs compared to untreated CSCs, except in prostate PC3 CSCs (Fig. 6c). Overexpression of the anti-apoptotic protein Bcl-xl in untreated CSCs confers chemo-resistance; however, the combinatorial treatment produced a significant decrease in protein expression levels, indicating sFRP4's pro-apoptotic capacity. Expression of the pro-apoptotic protein Bax was lower in untreated CSCs but increased significantly with combinatorial treatment. The increased Bax/Bcl-xl expression level ratio confirms the pro-apoptotic role of sFRP4.

Discussion

The role of CSCs in solid tumors is well established[20–22]. CSCs are in a quiescent state and remain in the resting phase of the cell cycle (G0 phase), expressing high levels of drug efflux transport systems[23,24]. Due to the CSCs' dormant state, chemotherapeutic drugs are unable to target CSCs, whereas they kill only proliferative tumor cells that are in M phase[25,26]. Therefore, the knowledge of chemo-resistance associated with CSCs is of great importance for our understanding of tumors of the reproductive system (i.e. breast, prostate, and ovary), which are tumors with poor prognosis. In order to gain further insights into the chemo-sensitization of CSCs, we investigated the role of sFRP4 when used in combination with the chemotherapeutic drugs doxorubicin and cisplatin on CSCs derived from various tumor cell lines. Our study demonstrates sFRP4 alone or in combinatorial treatment with chemotherapeutic drugs elicits anti-proliferative effects, spheroid disruption, decrease in cell survival, and initiation of apoptosis within the CSCs, therefore indicating chemo-sensitization.

The CSCs were identified from breast (MDA231 and MCF7), prostate (PC3 and LnCap), and ovary (A2780 P, A2780 ADR, and A2780 CIS) tumor cell lines based on their ability to form spheroids in serum free conditions, elevated expression of CSC surface markers, high expression of ABC drug transporters (ABCG2), cell survival protein (Cyclin D1), oncogenes (c-Myc), and the ability to escape cell death/apoptosis (Bcl-xl). The resistance in CSCs is often correlated to the CSC surface marker profiles; $CD44^{+High}$ and $CD133^{+High}$ cells are highly radio-resistant in colon cancer and they have a higher DNA repair capacity and ability to escape apoptosis compared to $CD44^{Low}$ and $CD133^{Low}$ CSCs[27]. Targeting $CD44^{+}$ cells demonstrated higher anti-proliferative activity *in-vitro* compared to the anti-tumor drug SN38 when used against colon, gastric, breast, esophageal, lung, and ovarian cancer cells[28,29]. A reduction in the expression of CSC markers was observed in our study, where we

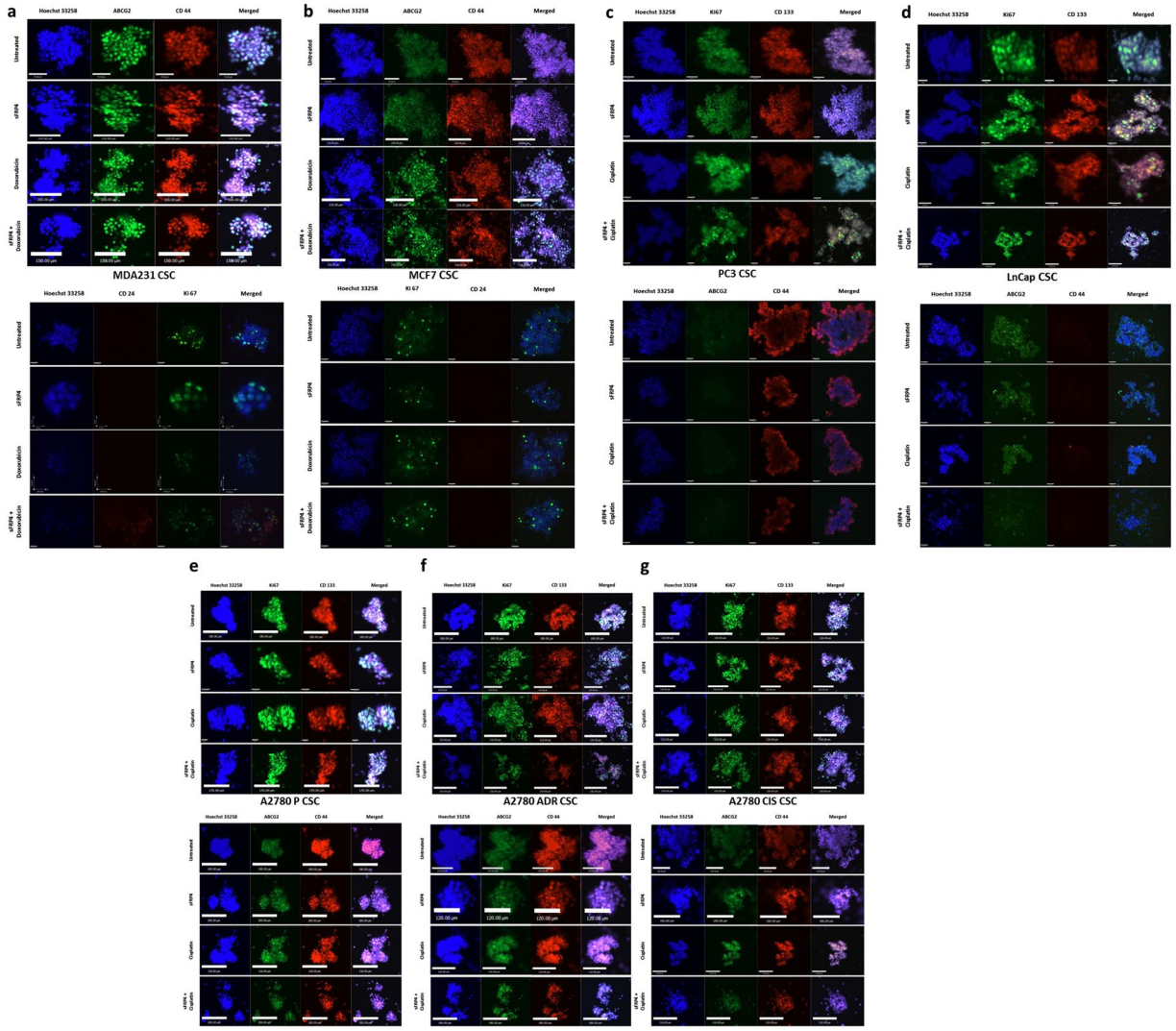

Figure 2. Effect of sFRP4 on CSC cell surface markers: Using Immunofluorescence, $CD44^+/CD24^-/CD133^+$/Ki67/ABCG2 were detected in all the tumor cell line-derived CSCs. sFRP4 in combination with doxorubicin/cisplatin showed disruption of the spheres. Nuclei were counterstained with Hoechst 33342 (blue). (**a,b**) $CD44^+/CD24^-$ breast tumor cell line CSCs (MDA231/MCF7). (**c,d**) Prostate tumor cell line derived CSCs. PC3 expressed $CD44^+/CD133^+$/Ki67/ABCG2. LnCap was negative for $CD44^+$. (**e–g**) $CD44^+/CD133^+$/Ki67/ABCG2 observed in ovary tumor cell line-derived CSCs (A2780 P/CIS/ADR). Immunofluorescence images are representative from 3 independent experiments. (Scale bar: 100 μm).

demonstrated that sFRP4 in combination with chemotherapeutic drugs was able to decrease the $CD44^+$ / 24^- population for breast-derived CSCs, and the $CD44^+$ / $CD133^+$ population for prostate and ovary-derived CSCs. In ovarian A2780 P derived CSCs, the cisplatin treatment aberrantly increased the $CD133^+$ $CD44^+$ population, and decreased the $CD44^+$ and $CD133^+$ alone population; this aberration could imply that phenotypic switching of CSCs has occurred, a process that is still not well understood[30].

Clinically, CSCs reside in anatomically distinct regions within the tumor microenvironment (known as niches), which preserve the CSCs' phenotypic plasticity, facilitate metastatic potential, and support high expression of drug efflux transporters, making them highly chemo-resistant[31]. Although CSC isolation and characterization has been studied extensively, *in-vitro* CSCs niches are characterized by spheroid forming capacity in serum free conditions[32]. We showed that targeting the Wnt signaling pathway by using sFRP4 has the capacity to disrupt the niches when sFRP4 was used in combination with chemotherapeutic drugs. Spheroid disruption by sFRP4 decreases the CSCs' plasticity and cell-cell adhesion, initiating the CSCs' differentiation towards tumor cells and reducing their self-renewal capacity. This opens the gateway for chemotherapeutic drugs to target the cells at high potency. We confirmed spheroid disruption using immunofluorescence, and we observed the spheroid disruption was associated with a reduced CSCs' marker profile. We also observed a marked reduction of the proliferation marker Ki-67 and drug transporter ABCG2 in sFRP4 combinatorial treatment. The prostate cell line LnCap-derived CSCs showed an absence of $CD44^+$ expression, which is in agreement with previous work[33].

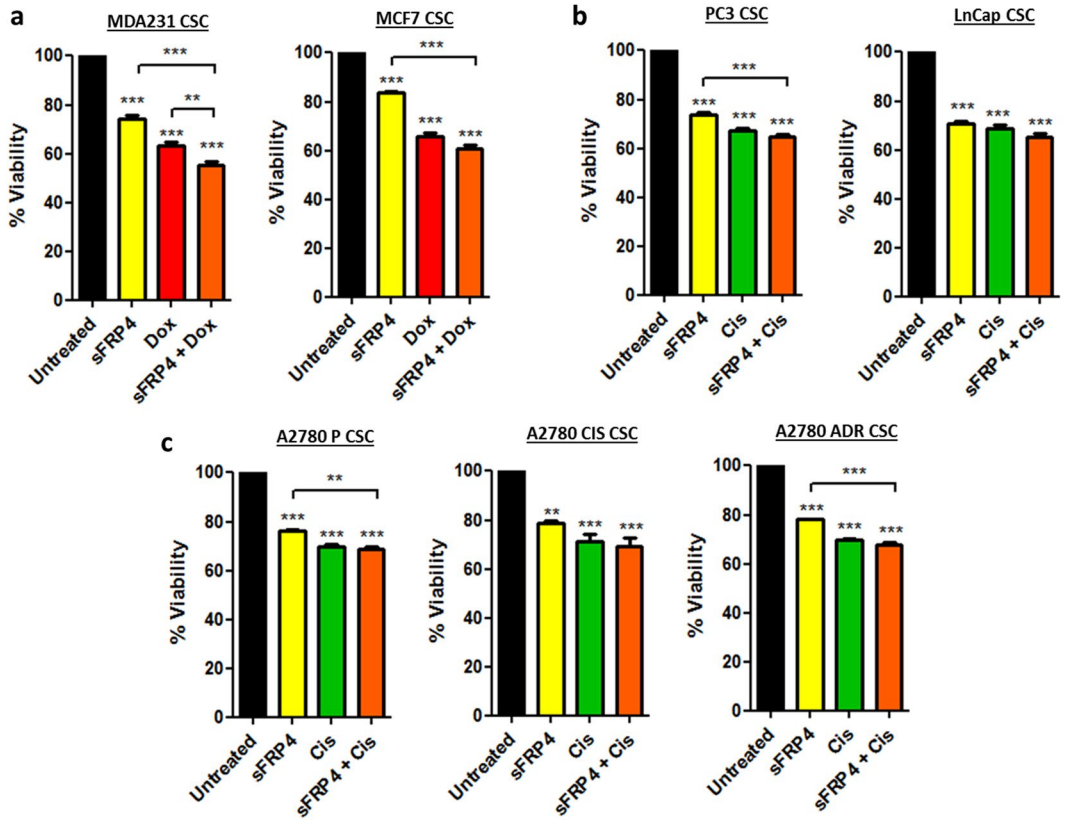

Figure 3. Effect of sFRP4 on CSC viability: Viability assay was performed by MTT after treatment of CSCs derived from (**a**) Breast tumor cell line-derived CSCs (MDA231/MCF7). (**b**) Prostate tumor cell line-derived CSCs (PC3/LnCap). (**c**) Ovary tumor cell line-derived CSCs (A2780 P/CIS/ADR) with sFRP4 alone or in combination with doxorubicin/cisplatin for 24 hr. Statistical analysis was performed using ANOVA for analysis variance with Bonferroni test for comparison showing significance as ***$P < 0.001$; **$P < 0.01$; *$P < 0.05$. Data are mean ± standard error of mean from 3 independent experiments.

In previous studies, sFRP4 has shown an anti-proliferative capacity in CSCs derived from glioblastoma multiforme, and head and neck tumor[19,34]. In this study we have demonstrated that sFRP4 in combination with chemotherapeutic drugs decreased the viability of CSCs compared to drug treatment alone, indicating sFRP4's role in the increased chemo-response of CSCs.

The Wnt signaling pathway has been reported to regulate stemness in CSCs derived from colon cancer[35] and breast cancer[36], although Wnt activation is higher in breast CSCs compared to normal stem-like cells[37]. Genes controlling the stemness of CSCs have distinct functions and are important for CSC development and self-renewal, and are responsible for replicative quiescence[38]. We hypothesized that cancer stemness inhibition can effectively suppress metastatic potential and tumor recurrence, although gene profiling of cancer stemness is more similar to embryonic stem cells than adult stem cells[39]. In this study, we show that sFRP4 reduced the expression of various stemness genes including *Sox2, Klf4, Nanog*, and *Oct4* when treated in combination with chemotherapeutic drugs. These genes encode key stemness transcription factors that are important for maintenance of pluripotency[40,41]. These data demonstrate the role of sFRP4 in inhibiting CSCs by modulating stemness gene expression.

Chemo-resistance of CSCs is due to alterations in expression of anti-apoptotic (Bcl-2 family) and pro-apoptotic (Bax) genes[42]. Apoptosis can be triggered by two pathways: (a) the extrinsic pathway where caspase-8 activation is initiated by the ligand of death receptors on the cell surface; and (b) the intrinsic pathway where mitochondria release *cytochrome c*[43]. Release of *cytochrome c* is a crucial step that activates caspase-9 by assembling the apoptosome, further activating the downstream executioners caspase 3/7[44]. In previous studies, the relationship between sFRP4 and apoptosis has been identified, demonstrating sFRP4 as a pro-apoptotic agent[18,45]. This was further confirmed by assessing the integrity of the mitochondrial membrane when the CSCs were treated with sFRP4. We observed the mitochondrial depolarization by application of the JC-1 assay, as low $\Delta\psi_M$ (mitochondrial membrane potential) due to depolarization is indicative of apoptosis, indicating sFRP4 chemo-sensitzation involves the initiation of apoptotic pathways. We further demonstrated the pro-apoptotic role of sFRP4 with an elevation in caspase 3/7 expression in CSCs treated with sFRP4 alone or in combination with chemotherapeutic drugs, indicating the later onset of apoptosis.

The overexpression of Bcl-2 is associated with chemo-resistance. The 3D protein structure of Bcl-xl revealed the structural similarities within the Bcl-2 family, possessing 4 BH domains and promoting cell survival by inactivation of Bcl-2 counterparts and preserving outer mitochondrial membrane integrity[46]. One of the early studies

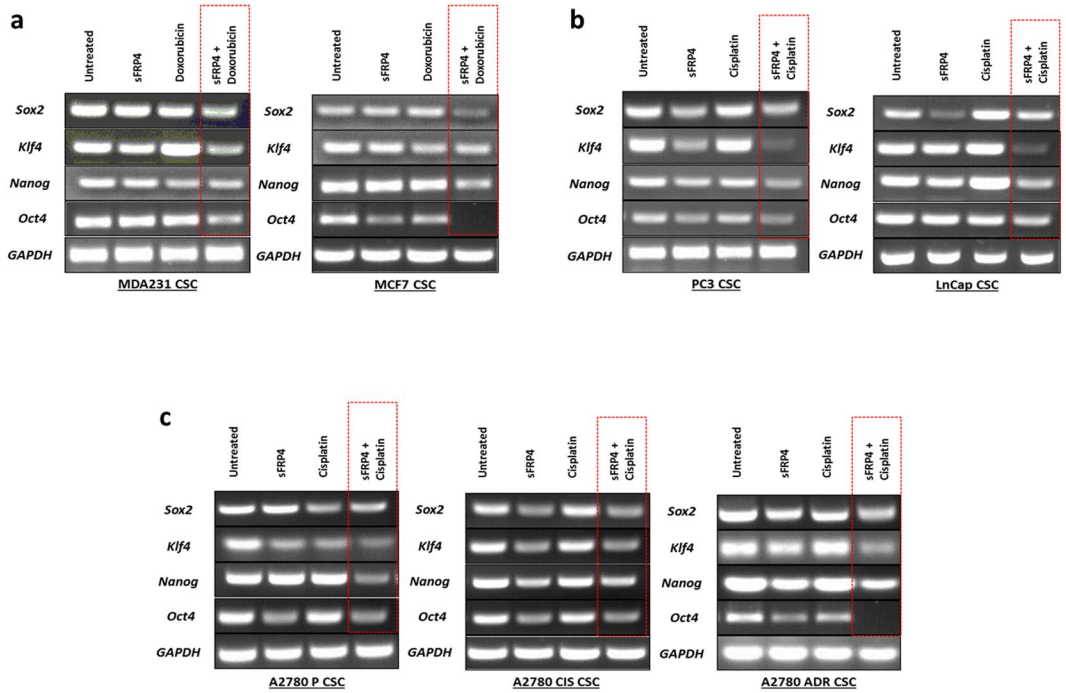

Figure 4. Effect of sFRP4 on CSC stemness gene expression: sFRP4 in combination with chemotherapeutic drugs (Dox/Cis.) reduced the expression of stemness-related genes, indicating loss of stem like expression and differentiation capacity. Semi-quantitative PCR images are representative of 3 experiments.

to show the chemo-sensitizing effects targeting Bcl-2 involved treating patients with Bcl-2 antisense (oblimersen sodium) in combination with chemotherapeutic drugs in chronic lymphocytic leukemia, leading to improved survival[47,48]. We hypothesized that sFPR4 has the potential to bind the hydrophobic groove of anti-apoptotic Bcl-2, which would oligomerize Bax and can subsequently lead to mitochondrial membrane potential depolarization, releasing *cytochrome c*. We demonstrated a gradual increase in Bax expression and decreased Bcl-xl expression with sFRP4 and chemotherapeutic drug treatment alone; however, the combinatorial treatment elevated Bax and inversed the effect on Bcl-xl. The Bax/Bcl-xl ratio is an indicator for apoptosis, and an increased ratio depicts the activation of caspase 3[49]. The Bax/Bcl-xl ratio was consistently high in all CSCs treated with combinatorial treatment. The elevated expression of apoptotic genes within all the CSCs indicates sFRP4's role as a pro-apoptotic agent.

We also observed a decrease in Cyclin D1 expression, which is an oncogene driving cell cycle progression. Cyclin D1 interacts with proteins involved with DNA repair, RNA metabolism, and cell structure; deregulation of Cyclin D1 will affect cellular processes and eventually lead to an inefficient DNA damage repair system[50]. In the Wnt signaling pathway, GSK-3β regulates Cyclin D1 degradation, and the gene expression is activated by Wnt signaling[51,52]. SFRP4 is a Wnt antagonist, which binds to the frizzled receptors and activates the GSK-3β destruction complex, initiating the degradation of Cyclin D1. In contrast, *c-Myc* expression showed no decrease in A2780 CIS, PC3, and MCF7-derived CSCs. c-Myc is an oncoprotein that is an important regulator in stem cell biology[53] and correlates to tumor metastasis[39]. CSCs exhibit a high expression of c-Myc, and downregulation of c-Myc leads to apoptosis under various circumstances[54–57]. We hypothesize that a different dose of sFRP4 and chemotherapeutic drugs would enable a reduction in *c-Myc* expression.

In summary, sFRP4 chemo-sensitizes CSCs derived from breast, prostate, and ovary tumor cell lines by reducing their pro-oncogenic profile, stemness capacity, cell survival protein and oncogene expression, making them more responsive to chemotherapeutic drugs. Chemo-sensitization by sFRP4 *in vivo* may decrease the required chemotherapeutic load required to reduce the tumor mass. SFRP4 prevents a sustained Wnt inhibition in order to provide a therapeutic window for chemotherapy while sparing normal Wnt-dependent tissues. Further *in vivo* studies may confirm the role of sFRP4 in the chemo-sensitization of CSCs to prevent tumor relapse and lead to tumor resolution.

Materials and Methods

Monolayer cell culture. Cell culture plates for adherent cells were purchased from Nunc™ (ThermoFisher Scientific). The human breast cells line MDA-MB 231 (ER-) and MCF-7 (ER+), human ovary cell lines A2780-P, A2780-ADR, and A2780-Cis, and human prostate cell lines PC-3 (AR−/PSA−) and LnCap (AR+) were purchased from American Type Culture Collection (ATCC, USA). The cells were cultured in RPMI-1640 medium (Gibco #11875–093) supplemented with 10% fetal bovine serum (Bovogen #SFBS) and 100 U/ml PenStrep (Life Technologies #15070063). All cells were maintained at 37 °C in a humid incubator with 5% CO_2.

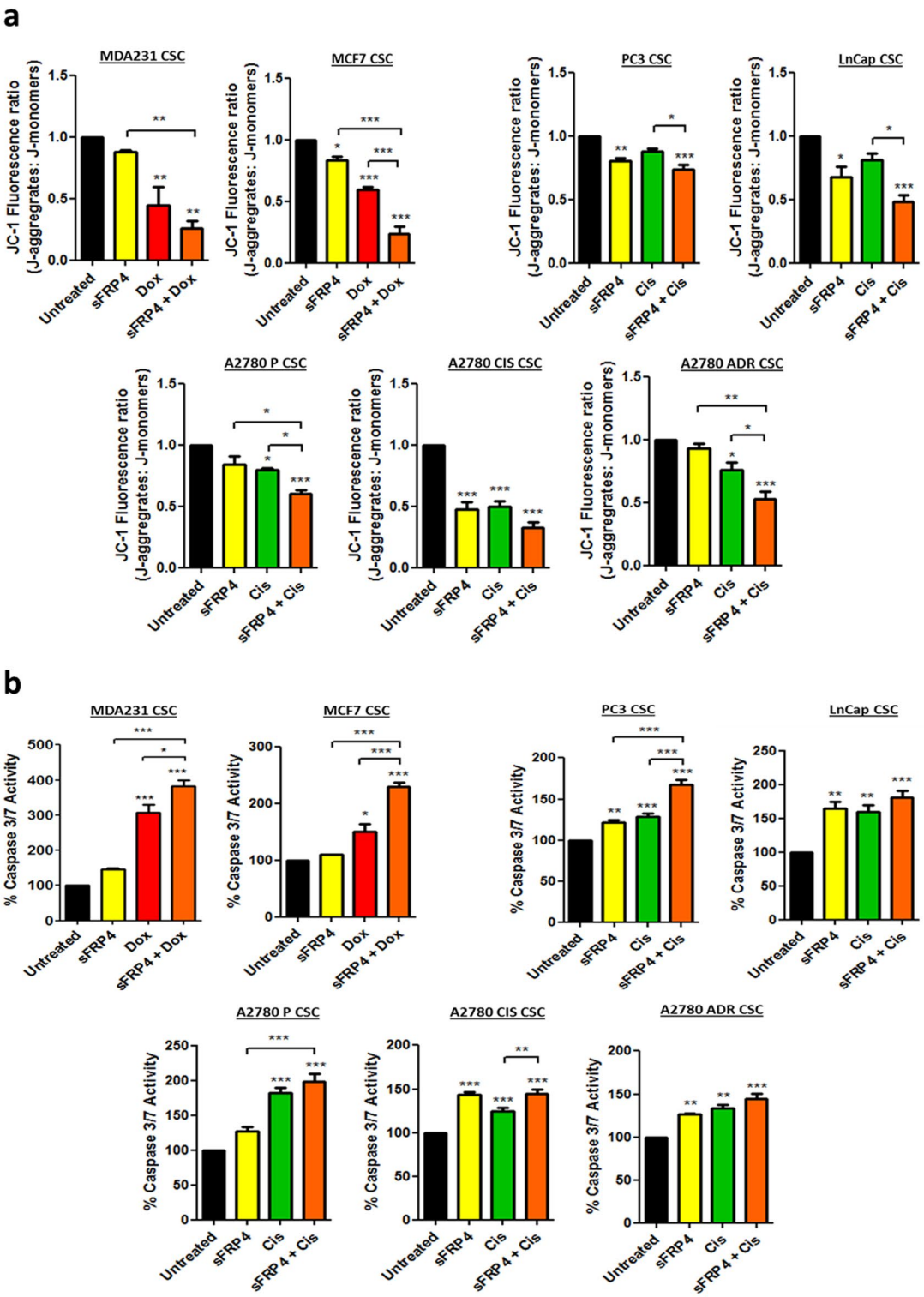

Figure 5. sFRP4 initiates early apoptotic events in CSCs. (**a**) Detection of JC-1: The JC-1 assay demonstrated a high mitochondrial depolarization in sFRP4, doxorubicin/cisplatin, and combination-treated CSCs, indicating early stage cell death and apoptotic response to the various drug treatments. The combinatorial treatment on CSCs showed maximum depolarization. (**b**) Detection of caspase-3 activity: Increasing amount of caspase-3 substrate indicates initiation of apoptosis. The caspase-3 activity (an indicator of late apoptosis) of CSCs was significantly upregulated in combinatorial treatment compared to untreated cells. Statistical analysis was performed using ANOVA for analysis variance with Bonferroni test for comparison showing significance as ***$P < 0.001$; **$P < 0.01$; *$P < 0.05$. Data are mean ± standard error of mean from 4 independent experiments.

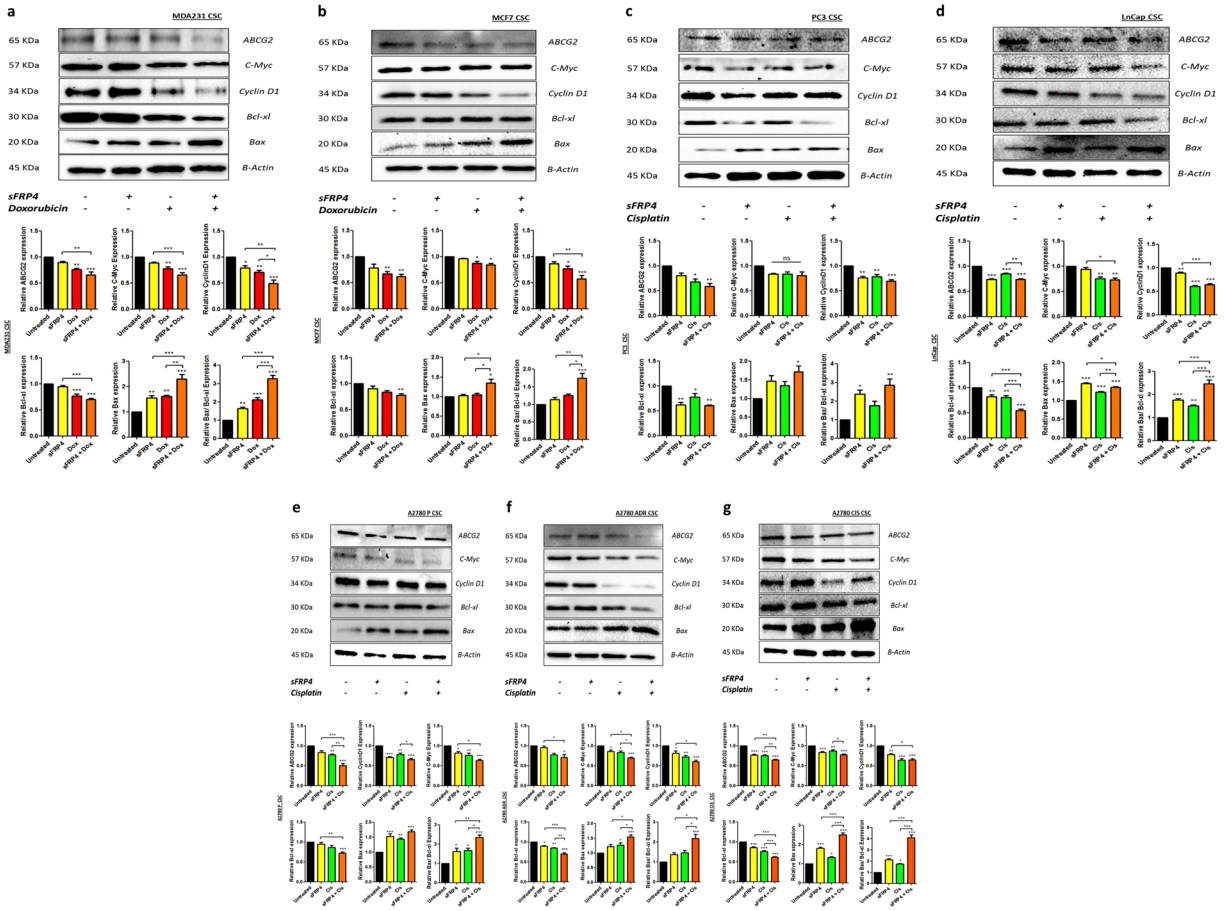

Figure 6. Effects of sFRP4 on protein expression levels: The combinatorial treatment of sFRP4 and chemotherapeutic drugs significantly upregulated the apoptotic protein (Bax) and downregulated cell survival (Cyclin D1) and oncogenes (*C-Myc*). (**a,b**) Breast tumor cell line-derived CSCs (MDA231/MCF7). (**c,d**) Prostate tumor cell line-derived CSCs (PC3/LnCap). (**e–g**) Ovary tumor cell line-derived CSCs (A2780 P/CIS/ADR). In combinatorial treatment, an elevated Bax/Bcl-xl ratio corresponded to elevated cell apoptosis. Statistical analysis was performed using ANOVA for analysis variance with Bonferroni test for comparison showing significance as $^{***}P < 0.001$; $^{**}P < 0.01$; $^{*}P < 0.05$. Blots and relative protein expressions are mean ± standard error of mean from 3 independent experiments.

Cancer stem cell isolation. For CSC isolation, culture plates with an ultra-low-attachment surface were purchased from Corning Life Sciences. CSCs were cultured in serum-free medium (SFM) containing basal medium RPMI-1640 + DMEM-HG (HyClone, USA #SH30081.02) supplemented with the growth factors bFGF (20 ng/ml) (ProSpec Bio #cyt-085), EGF (20 ng/ml) (ProSpec Bio #cyt-217), and 1× B27 (Gibco #17504044), and 100 U/ml PenStrep (Life Technologies #15070063). CSC-enriched populations of cells were obtained by plating a single cell suspension of breast, ovary, and prostate tumor cells at 10000 cells/cm² in SFM on Low-adherent six-well plates (Corning #3471). CSCs were isolated in SFM; the spheroids are formed at 3rd Day of plating tumour cells. SFRP4 (250 pg) and chemotherapeutic drugs alone or in combination were added on the 3rd Day for 24 hours. Post 24 hour treatment; the spheroids were dissociated and maintained in CSC culture medium in low-attachment-surface plates for functional studies.

Chemo-Sensitization/Drug treatment. The drugs used in this study were purified sFRP4 (R&D Systems #1827-SF-025), doxorubicin (Sigma #D1515), and cisplatin (Sigma #P4394). CSC sensitization with sFRP4 was performed by adding sFRP4 to the cell culture at 250 pg/ml[58] for 24 hr at 37 °C in 5% CO_2 incubator. Doxorubicin was tested at an IC50 value of 1–10 μM. Cisplatin was tested at its IC50 value of 10–50 μM. The drug treatment for the downstream analysis was optimized at 250 pg of sFRP4 alone or in combination with 5 μM of doxorubicin (breast tumor cells only) and 30 μM of cisplatin (ovary and prostate tumor cells only) for 24 hr. An MTT assay was used for the analysis of cellular viability.

Cell Surface Markers. To assist in determining their identity, cell surface markers (Table 2) were examined in both monolayers and CSCs by flow cytometry (BD FACSCANTO II) using CellQuest data acquisition and analysis software. APC-CD44 (1:100) (BioLegend #338805), PE Cy7-CD24 (1:10) (BioLegend #311119), and PE-CD133 (1:100) (BioLegend #372803). Cells incubated with conjugated irrelevant IgGs were used as negative controls.

Cancer	CSC Markers	Reference
Breast	$CD24^{-/low}/CD44^{+}$	3, 59
Ovary	$CD133^{+}/ALDH1^{+}$	60
Prostate	$CD133^{+}/CD44^{+}/ABCG2^{+}$	61

Table 2. Tumor specific cancer stem cell markers.

Immunofluorescence staining. The spheroids were plated on Poly-D-lysine pre-coated 96 well plates (Sigma #6407), incubated at 37 °C for 3 hr, and then fixed in 4% paraformaldehyde (Sigma #P6148) overnight at 4 °C. The cells were washed three times with PBS, incubated for 1 hr in 1% BSA Blocking buffer, and incubated with primary antibodies CD44 (Cell Signaling #3570); CD24 (ThermoFisher #MA5-11828); CD133/1 (Miltenyi #130-090-422); ABCG2 (Cell Signaling #42078); Ki-67 (Millipore #AB9260) overnight at 4 °C. After three 10 mins washes with PBS, the cells were incubated with the appropriate secondary antibody (see Supplementary Table 1) for 1 hr at room temperature. After three 10 min PBS washes, cells were incubated with Hoechst 33342 (Sigma #14533) at 1:20000 dilution at room temperature for 15 min. The cells were then washed with PBS three times for 5 min each and observed using an Ultraview Vox spinning disk confocal microscope (Perkin Elmer).

MTT Viability Assay. *Cell viability kit.* An MTT kit (Sigma #M5655) was used to measure cell metabolic viability.

Monolayers. 5000 cells/cm² were plated in a flat-bottomed 96-well plate for 2 days with culture medium. After that, sFRP4 alone or in combination with the tumor-specific drug was added and the cells were incubated for 24 hr. After drug treatment, the MTT assay was performed as per the manufacturer's instructions.

CSCs. 10000 cells/cm² of monolayer cells were plated in a low-adherent flat-bottomed 96-well plate (Corning #3474) for 3 days in non-adherent SFM conditions. After that, sFRP4 alone or in combination with the tumor-specific drug treatment was added and the cells left for 24 hr, following which the MTT assay was performed. Plates were read at 595 nm using an EnSpire Multilabel Plate Reader (Perkin-Elmer).

Reverse transcription-polymerase chain reaction. Total RNA was isolated from cells using TRIzol reagent (Life Technologies #15596026) followed by chloroform extraction, isopropanol precipitation, and a 75% (v/v) ethanol wash. RNA samples (1 μg) were reverse-transcribed to cDNA using a High Capacity cDNA kit (Applied Biosystems #4368814). cDNA in 1 μl of the reaction mixture was amplified with PCR Master Mix (Life Technologies #K0171) and 10 μmol each of the sense and antisense primers. The thermal cycle profile was as follows: denaturation at 95 °C for 30 s, annealing at 55–61 °C for 30 s depending on the primers used, and extension at 72 °C for 90 s. Each PCR reaction was carried out for 35 cycles, and the PCR products were size fractionated on 1% agarose gel/GelGreen (1:10000) (Fisher Biotec #41005) and visualized under UV Trans illumination (FB Biotech). The primer sequences are described in Supplementary Table 2.

Western Blotting. Cells were washed twice with PBS and then lysed in RIPA lysis buffer (Sigma #R0278) (150 mM NaCl, 1.0% IGEPAL® CA-630, 0.5% sodium deoxycholate, 0.1% SDS, 50 mM Tris, pH 8.0, Proteinase Inhibitor 1x). Post sonication, cell lysates were centrifuged at 14000 g for 10 min at 4 °C, and the supernatants were used for Western blotting. The lysates were resolved by sodium dodecyl sulfate-polyacrylamide gel electrophoresis, transferred onto nitrocellulose membranes, and then stained with 0.1% Ponceau S solution (Sigma #P3504) to ensure equal loading of the samples. After being blocked with 5% non-fat milk for 60 min, the membranes were incubated with primary antibodies ABCG2 (Cell Signaling #42078); c-Myc (Cell Signaling #5605); Cyclin D1 (Abcam #ab134175); Bcl-xL (Cell Signaling #2764); Bax (Cell Signaling #5023); β-Actin (Cell Signaling #4970) overnight, and the bound antibodies were visualized with horseradish peroxidase-conjugated secondary antibodies using the ECL Western Blotting Substrate (Amersham, GE #RPN2106) on a Chemi-Doc (Bio-Rad) imaging analyzer. The Primary antibodies concentrations are described in Supplementary Table 3.

Apoptosis Assays. *JC-1 Assay.* $\Delta\psi_M$ is an important parameter of mitochondrial membrane and has been used as an indicator of cell health. JC-1 enters the mitochondria and changes its fluorescent properties based on aggregation of the probe, and forms complexes known as J-aggregates with intense red fluorescence. High $\Delta\psi_M$ predicts healthy cells and low $\Delta\psi_M$ exhibits mitochondrial membrane potential depolarization indicative of apoptosis. JC-1 activity was measured using JC-1 Mitochondrial Membrane Potential Assay Kit (Cayman #10009172). CSCs were enriched in 96 well low attachment plates, and treated with sFRP4 alone and in combination with chemotherapeutic drugs for 24 hr and compared with untreated CSCs. Post-treatment, 10 μl of JC-1 staining solution was added to each well and incubated at 37 °C for 30 min. The plate was washed with assay buffer at 400 g for 5 min twice. This was followed by addition of 100 μl of assay buffer to each well and read using an EnSpire Multilabel Plate Reader (Perkin-Elmer) for analysis. JC-1 aggregates were measured at excitation and emission wavelengths of 535 nm and 595 nm respectively. JC-1 monomers were measured at excitation and emission wavelengths of 485 nm and 535 nm. The ratio of fluorescent intensity of J-aggregates and J-monomers (Red: Green) was used as an indicator of cell health. The JC-1 assay kit is highly light sensitive and all procedures were conducted in dark conditions.

Caspase-3 Assay. Caspase-3 activity was measured using the EnzChek Caspase-3 Assay Kit II (Molecular Probes #E13184). Briefly, 50 µl of the supernatant was added to an individual well of a 96-well micro fluorescent plate and incubated for 10 min at room temperature. After incubation, 50 µl of the 2× working substrate (5 M Z-DEVD-R110) were added to each well and further incubated for 30 min at 37 °C. Fluorescence was measured at 485 nm excitation and 538 nm emissions using an EnSpire Multilabel Plate Reader (Perkin-Elmer). Caspase-3 activity was expressed as arbitrary units of fluorescence.

Statistics. Statistical analysis was performed with GraphPad Prism V5.0 (GraphPad software, La Jolla, USA) using one-way ANOVA for analysis variance with Bonferroni test for comparison showing significance as ***$P < 0.001$; **$P < 0.01$; *$P < 0.05$. Data are presented as mean ± standard error of mean.

Data Availability. The datasets generated during and/or analysed during the current study are available from the corresponding author on reasonable request.

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Author Contributions

Abhi D. drafted the outline and generated the data. S.K. contributed the viability data. Abhi D. wrote the manuscript. Abhi D. and Arun D. conceived the study, and Arun D., F.A., and P.N. critically reviewed, revised, and approved the final manuscript.

Additional Information

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Title: Secreted Frizzled-related protein 4 (sFRP4) chemo-sensitizes cancer stem cells derived from human breast, prostate, and ovary tumor cell lines

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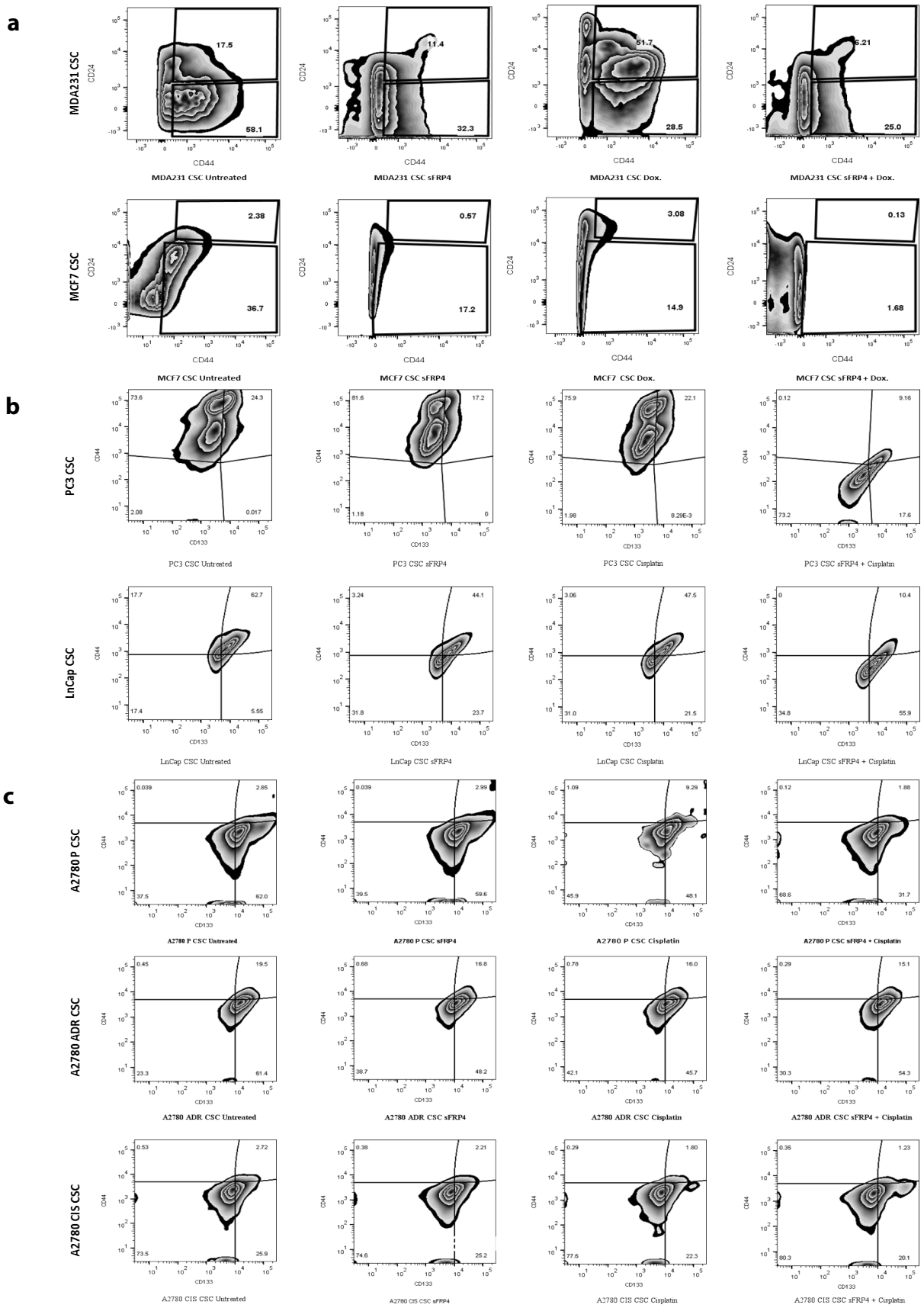

Supplemetary Figure 1: Effect of sFRP4 on CSC characterisation: Using flow cytometry A) CD44/CD24 were used to detect breast-derived CSCs, B/C) CD133/ CD44 for ovary and prostate-derived CSCs. sFRP4 in combination with Doxorubicin/ Cisplatin reduced the CD44/24/133 CSC population.

Supplementary Table 1: Primary antibodies used for Immunofluorescence

Primary Antibody (Clone)	1° Dilution	Catalogue No.	Secondary Antibody	2° Dilution
CD44 (156-3C11)	1:300	Cell Signaling #3570	Anti-mouse IgG, HRP-linked	1:10000
CD24 (SN3)	1:300	ThermoFisher #MA5-11828	Anti-mouse IgG, HRP-linked	1:10000
CD133/1 (AC133)	1:100	Miltenyi #130-090-422	Anti-rabbit IgG, HRP-linked	1:10000
ABCG2 (D5V2K)	1:300	Cell Signaling #42078	Anti-rabbit IgG, HRP-linked	1:10000
Ki-67 (na)	1:500	Millipore #AB9260	Anti-rabbit IgG, HRP-linked	1:10000
Hoechst 33342	1: 20000	Sigma #14533	-na-	-na-

Supplementary Table 2: Primer sequences for CSC stemness markers

Genes	Primers	Base Pair (bp)	Annealing Temperature °C
Sox2	F- 5’ CCATCCACACTCACGCAAAA 3’ R- 5’ TATACAAGGTCCATTCCCCCG 3’	139	59
Oct4	F- 5’ TCCCATGCATTCAAACTGAGG 3’ R- 5’ CCAAAAACCCTGGCACAAACT 3’	103	60
Nanog	F- 5’ TGGACACTGGCTGAATCCTTC 3’ R- 5’ CGTTGATTAGGCTCCAACCAT 3’	142	59
KLF4	F- 5’ CTGCGGCAAAACCTACACAA 3’ R- 5’ GGTCGCATTTTTGGCACTG 3’	182	60
GAPDH	F- 5’ CAGAACATCATCCCTGCATCCACT 3’ R- 5’ GTTGCTGTTGAAGTCACAGGAGAC 3’	185	61

Supplementary Table 3: Primary and secondary antibodies used in Western blotting to measure relative protein expression

Primary Antibody (Clone)	1° Dilution	Molecular Weight (Kda)	Catalogue No.	Secondary Antibody	2° Dilution
ABCG2 (D5V2K)	1:1000	65-80	Cell Signaling #42078	Anti-rabbit IgG, HRP-linked	1:2000
c-Myc (D84C12)	1:1000	57-65	Cell Signaling #5605	Anti-rabbit IgG, HRP-linked	1:2000
Cyclin D1 (EPR2241)	1:10000	34	Abcam #ab134175	Anti-rabbit IgG, HRP-linked	1:2000
Bcl-xL (54H6)	1:1000	30	Cell Signaling #2764	Anti-rabbit IgG, HRP-linked	1:2000
Bax (D2E11)	1:1000	20	Cell Signaling #5023	Anti-rabbit IgG, HRP-linked	1:2000
β-Actin (13E5)	1:1000	45	Cell Signaling #4970	Anti-rabbit IgG, HRP-linked	1:2000

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Regulation of Cancer Stem Cell Metabolism by Secreted Frizzled-Related Protein 4 (sFRP4)

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Abstract: Tumours contain a small number of treatment-resistant cancer stem cells (CSCs), and it is through these that tumour regrowth originates at secondary sites, thus rendering CSCs an attractive target for treatment. Cancer cells adapt cellular metabolism for aggressive proliferation. Tumour cells use less efficient glycolysis for the production of ATP and increasing tumour mass, instead of oxidative phosphorylation (OXPHOS). CSCs show distinct metabolic shift and, depending on the cancer type, can be highly glycolytic or OXPHOS dependent. Since Wnt signalling promotes glycolysis and tumour growth, we investigated the effect of the Wnt antagonist secreted frizzled-related protein 4 (sFRP4) on CSC metabolism. We demonstrate that sFRP4 has a prominent role in basal glucose uptake in CSCs derived from breast and prostate tumour cell lines. We show that sFRP4 treatment on CSCs isolated with variable glucose content induces metabolic reprogramming by relocating metabolic flux to glycolysis or OXPHOS. Altogether, sFRP4 treatment compromises cell proliferation and critically affects cell survival mechanisms such as viability, glucose transporters, pyruvate conversion, mammalian target of rapamycin, and induces CSC apoptosis under conditions of variable glucose content. Our findings provide the feasibility of using sFRP4 to inhibit CSC survival in order to induce metabolic reprogramming in vivo.

Keywords: cancer stem cells; metabolism; secreted fizzled-related protein; glycolysis; glucose; apoptosis

1. Introduction

Accumulating evidence suggest that tumours of various tissue origins, including breast, prostate, and ovary contain a small sub-population of cells with stemness capacity, often referred to as cancer stem cells (CSCs) or tumour initiating cells [1–4]. In addition to the CSCs' self-renewal and migratory capacity, they also possess the ability to efflux toxic compounds and chemotherapeutic agents due to their high expression of ATP-dependent efflux pump ABCG2, high DNA repair system, and activation of survival cascades [5–7].

Current research has established certain key components and signalling pathways that affect the stemness and differentiation of CSCs [8–11], although the effect of nutrients and metabolites on CSCs remains elusive. A recent study suggests that CSCs have particular metabolic properties enabling their identification from the bulk tumour cells based upon their biochemical profile [12]. Another study demonstrated that brain CSCs exhibit low mitochondrial respiratory activity and prefer a hypoxic environment to maintain their stemness [11]. Glioma stem cells (GSCs) were glycolysis driven and were intrinsically sensitive to the use of a glycolytic inhibitor [13]. However, cancer cells prefer glycolysis for their ATP production, and CSCs appear to have higher glycolytic activity. The Warburg hypothesis is consistent with the CSCs' dependency on glycolysis and switching on oxidative phosphorylation to facilitate cytosolic glycolysis [14,15]. Based on these observations that glucose is an essential nutrient

for CSCs, we reasoned that glucose might have a significant effect on the CSC subpopulation in bulk tumour cells. Furthermore, this enabled us to evaluate CSC survival under conditions of variable glucose content, and we also investigated the role of Wnt antagonism in regulating CSC survival under these conditions.

Wnt signalling plays an important role in tissue development and maintenance of normal tissues, though aberrant Wnt signalling activation is implicated in many cancers [16]. Wnt signalling in the "ON" state leads to active β-catenin accumulation in the nucleus, and its interaction with LEF/TCF (lymphoid enhancer factor/T-cell factor) transcription factors leads to activation of Wnt target genes that are important for cancer cell survival. Aberrant Wnt signalling has been implicated in tumorigenesis, as its downstream targets are involved in cell survival, differentiation, and proliferation; therefore, inhibition of the Wnt pathway is a potential strategy for halting tumour progression. Secreted frizzled-related protein 4 (sFRP4) is a Wnt antagonist inhibiting canonical Wnt signalling by binding to Wnt ligands and frizzled receptors [17]. Our previous studies have identified sFRP4's ability to inhibit multiple functional outputs of oncogenic Wnt signalling in CSCs, including a decrease in viability and epithelial-mesenchymal transition (EMT) induction, inhibiting angiogenesis, inducing apoptosis, and modulating cell survival [8,18]. Here, we propose a novel function of sFRP4 in the regulation of CSC metabolism.

In this study, we used cell lines from breast (MDA231 and MCF7) and prostate (PC3 and LnCap) tumours to isolate CSCs as an in vitro model of endocrine-related tumours. We investigated the effect of sFRP4 on CSCs isolated in culture medium with no, low, and high glucose content. The addition of glucose to the culture medium induced a significant increase in CSC viability, which was decreased post sFRP4 treatment under the same conditions. We also demonstrated that the CSC metabolic profile changes with increasing glucose content in culture medium, and Wnt signalling plays a key role in mediating glucose induced CSC survival. Finally, we investigated the potential therapeutic effect of sFRP4 on CSC viability, glucose uptake, glutamine uptake, glutamate secretion, NAD+/NADH ratio, and metabolically relevant proteins, and showed that sFRP4 compromised CSC viability and impaired CSC survival by initiating apoptosis regardless of glucose content.

2. Results

2.1. The Sphere Forming Capacity of CSCs Is Reduced by sFRP4 Irrespective of Glucose Content

To investigate the potential phenotypic changes that glucose dependency might induce in CSCs, we evaluated CSC morphology in different culture media containing varied glucose levels. CSCs were treated with sFRP4 (250 pg) for 24 h. The untreated spheroids remained intact, whereas the sFPR4 treated cells showed disruption of spheroids (Figure 1) in No Glucose/Low Glucose/High Glucose culture medium. However, sFRP4 segregated the spheroids in MCF-7, PC-3, and LnCap CSCs (Figure 1). Without glucose, CSCs spheroids are more stressed and susceptible to sFRP4's segregation capacity. CSCs isolated in low glucose culture medium showed a similar effect as no glucose, indicating that glucose is critical for CSC survival. However, sFRP4 segregated the spheroids in all glucose conditions, indicating that sFRP4's effects are not influenced by glucose conditions.

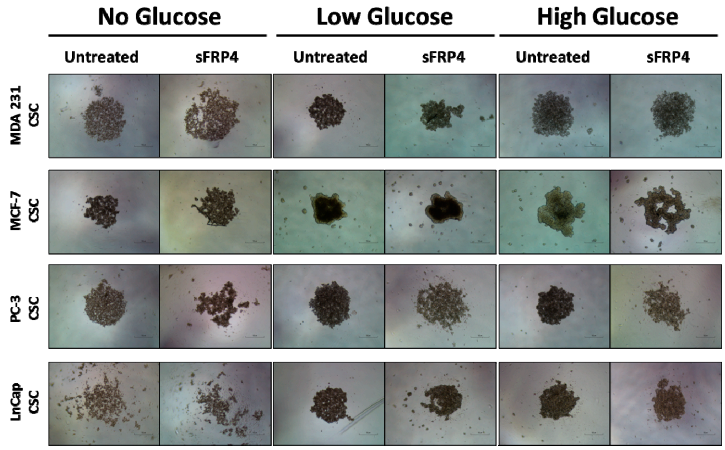

Figure 1. Effect of Secreted Frizzled Related Protein-4 on Cancer Stem Cells morphology: CSCs were isolated from breast and prostate tumour cell lines with increasing glucose concentrations and treated with sFRP4 (250 pg). The sFRP4 treatment results in disruption of the CSC sphere. (Scale bar: 100 µm). Images are representative of all the experiments.

2.2. CSC Viability Is Reduced by sFRP4 in Low and High Glucose Conditions

Using a Cell Counting Kit-8 assay, it was observed that the viability of CSCs increased with increasing glucose content in the culture medium (Figure 2).

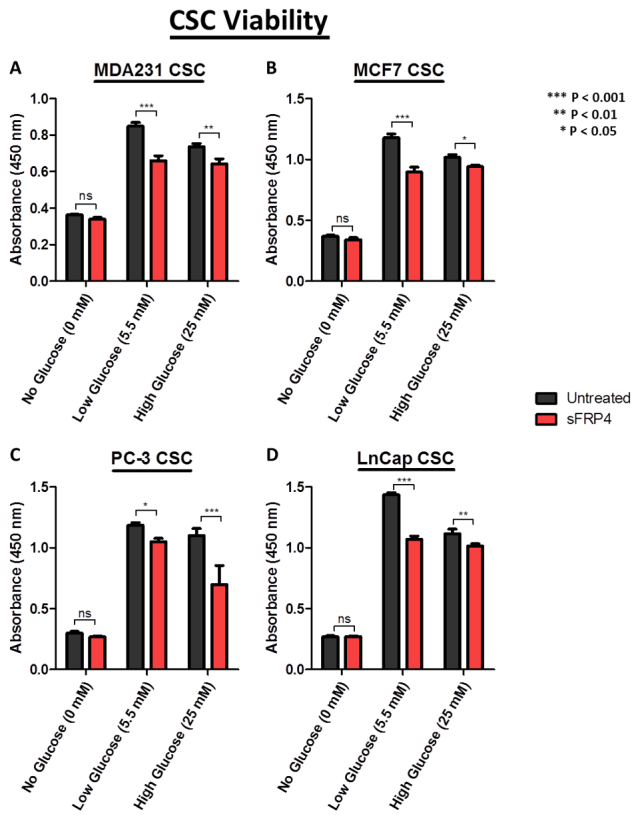

Figure 2. Effect of sFRP4 on CSC viability: Viability assay was performed using Cell Counting Kit-8 after treatment of CSCs derived from (**A**) MDA231; (**B**) MCF7; (**C**) PC-3; and (**D**) LnCap cell lines treated with sFRP4 for 24 h. Statistical analysis was performed using ANOVA for analysis variance with Bonferroni test for comparison showing significance as *** $p < 0.001$; ** $p < 0.01$; * $p < 0.05$. Data are mean ± standard error of mean from three independent experiments.

This indicates the requirement of glucose for CSC metabolism and survival. However treatment with sFRP4 significantly inhibited the viability of CSCs in low and high glucose conditions compared to untreated CSCs. In low glucose conditions, sFRP4 critically affected the viability in MDA231 (Figure 2A), MCF-7 (Figure 2B), PC3, and LnCap CSCs (Figure 2D), whereas a minimal effect was observed in no-glucose conditions for all CSCs.

2.3. Correlation Coefficient of sFRP4 with Metabolic Gene-Set

Our initial goal was to generate a gene-expression signature representing sFRP4 activation and to use that signature to identify metabolic targets in breast and prostate cancer through The Cancer Genome Atlas. The set of genes was further narrowed by identifying the subset of genes with established or putative roles in cancer metabolism. Because some of the genes were identified as cancer amplified genes, we further analyzed the gene set to identify putative metabolism driver genes. This was done by calculating the Spearman and Pearson correlation coefficient between the mRNA expression values from The Cancer Genome Atlas (TCGA) patient tumour data. Correlation coefficients were calculated for both breast (Figure 3A) and prostate (Figure 3B) cancer, and average correlations were calculated for each gene. The gene set comprised metabolic genes such as *AMPKB1* (AMP Kinase), *mTOR* (mammalian target of rapamycin), *GLUT1* (glucose transporter), *SLC1A5* (glutamine transporter), *BAD* (Bcl-2 associated death promotor), and *PDHA1* (pyruvate dehydrogenase). The analysis revealed a negative correlation coefficient between *sFRP4* and the gene set, indicating an inhibitory effect of *sFRP4* on all those genes. The only gene to have a positive correlation coefficient was *BAD*, indicating the pro-apoptotic capacity of sFRP4 in cancer metabolism in both the tumours examined. Although *AMPK* showed minimal correlation, it plays an important role in regulating the PI3K/AKT/mTOR signalling cascade, and we decided to include this gene set for our protein modification study.

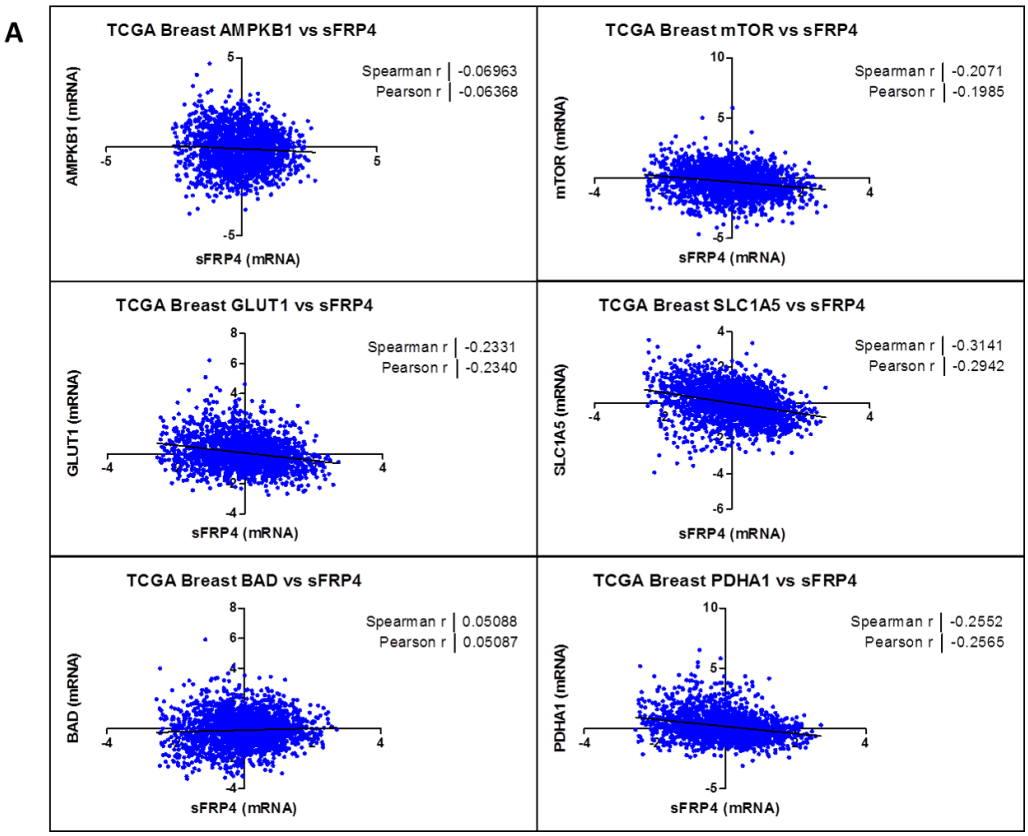

Figure 3. *Cont.*

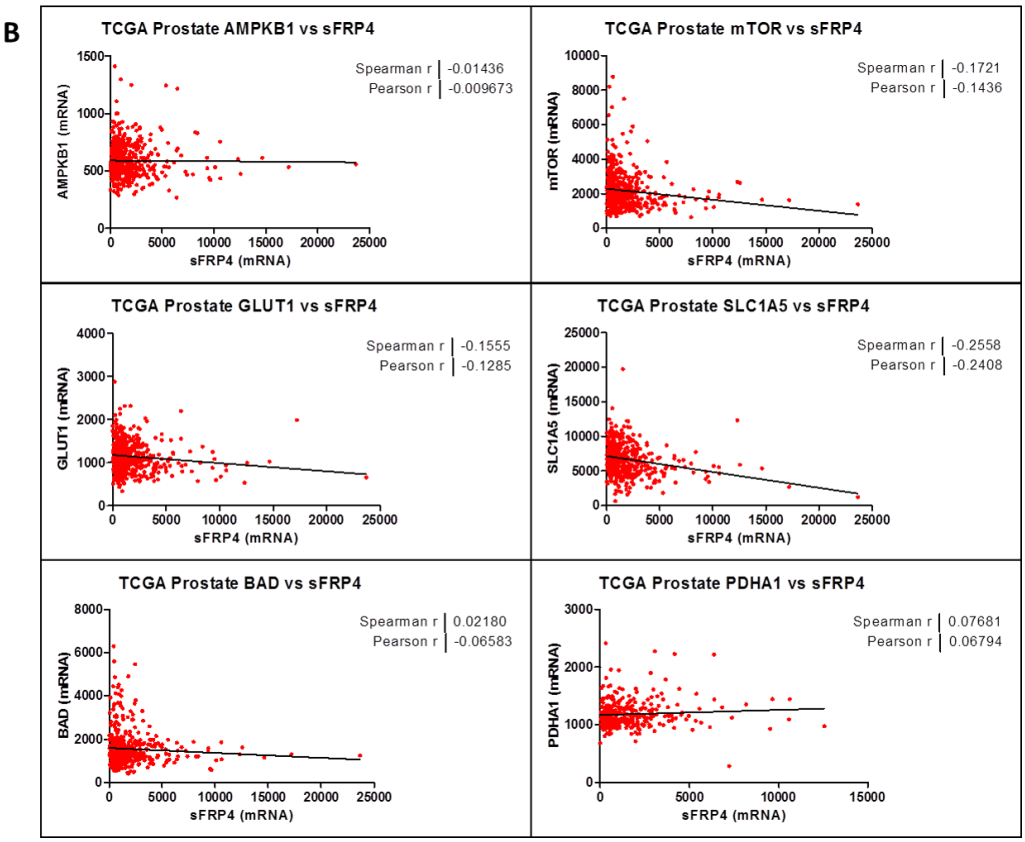

Figure 3. Correlation coefficient of sFRP4 with metabolic gene-set: Spearman and Pearson correlation coefficient between the mRNA expression values from TCGA patient tumour data. (**A**) Breast Invasive Carcinoma (n = 825) and (**B**) Prostate Adenocarcinoma (n = 333).

2.4. The Effect of Glucose and sFRP4 on Glucose-Uptake in CSCs

To better understand the effect of glucose on glycolytic metabolism, the CSCs were isolated in various glucose concentrations, and cellular uptake was detected with a glucose uptake-glo assay (Figure 4). Glucose induced an increase in glucose uptake by 2-fold in MDA231 CSCs, 5 folds in MCF7 CSCs and PC3 CSCs low glucose groups, whereas in the high glucose groups there was a 2 folds increase for MDA231 CSCs and LnCap CSCs, and 6-fold in PC3 CSCs, compared with the no glucose groups (Figure 4A–D). Breast CSCs had higher glucose uptake in the low glucose groups (Figure 4A,B); whereas prostate CSCs had higher glucose uptake in the high glucose groups (Figure 4C,D). We then monitored the effect of sFRP4 on glucose uptake. The levels of glucose uptake in low glucose groups significantly increased in all CSCs, whereas they decreased significantly in high glucose groups, suggesting that sFRP4 has a direct effect on glycolytic flux and in increasing the glycolytic activity.

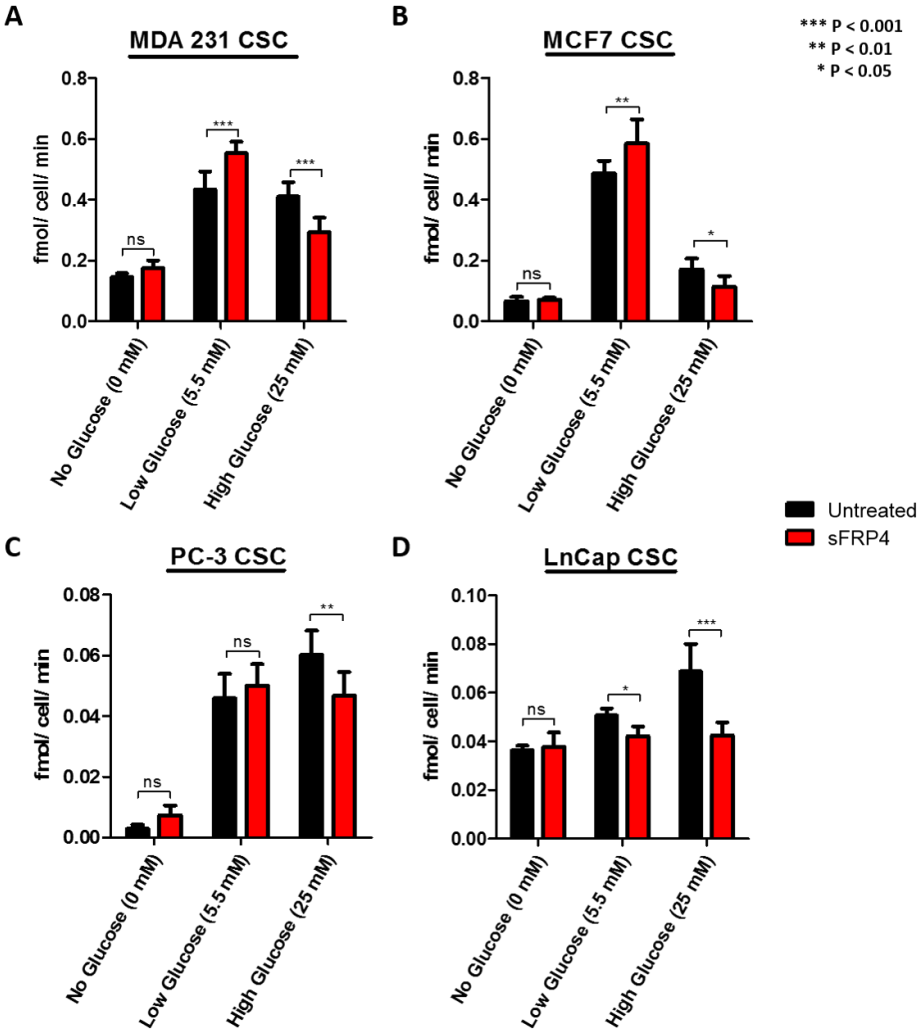

Figure 4. The effect of sFRP4 on glucose-uptake in CSCs: CSCs were isolated in various glucose concentrations and treated with sFRP4 (250 pg) for 24 h. Cellular uptake was detected with Glucose Uptake-glo assay. (**A**) MDA231 CSCs; (**B**) MCF7 CSCs; (**C**) PC-3 CSCs; and (**D**) LnCap CSCs. Statistical analysis was performed using ANOVA for analysis variance with Bonferroni test for comparison showing significance as *** $p < 0.001$; ** $p < 0.01$; * $p < 0.05$. Data are mean ± standard error of mean from three independent experiments.

2.5. Changes in Extracellular Metabolites with sFRP4 Treatment of CSCs

To further characterize the metabolic profiles of CSCs, changes in extracellular metabolite levels during CSC growth in culture were measured. The starting concentration of glutamine is consistent in all CSCs medium, and as cells grow they consume these metabolites. During growth, glutamate is secreted and accumulated in the medium. The CSCs consumed glutamine and secreted glutamate with time and cell density dependence. Glutamine consumption was more robust in CSCs isolated in all glucose groups (Figure 5A–D). The glutamine uptake increased with increasing glucose content in the medium. In contrast, glutamate secretion (Figure 5E–H) was also observed in all CSCs, indicating glutaminolysis activity. The relative luminescence units (RLU) increased in glutamine uptake with increase in glucose content, indicating the co-activity of glycolysis and glutaminolysis. However, glutamate secretion was significantly different in all the CSCs. Upon addition of sFRP4, we observed a decrease in glutamine uptake and glutamate secretion in all CSCs. However, PC3 CSCs (Figure 5C, G)

exhibited a marked effect following sFRP4 treatment, as we observed a higher inhibition of extracellular metabolite secretion.

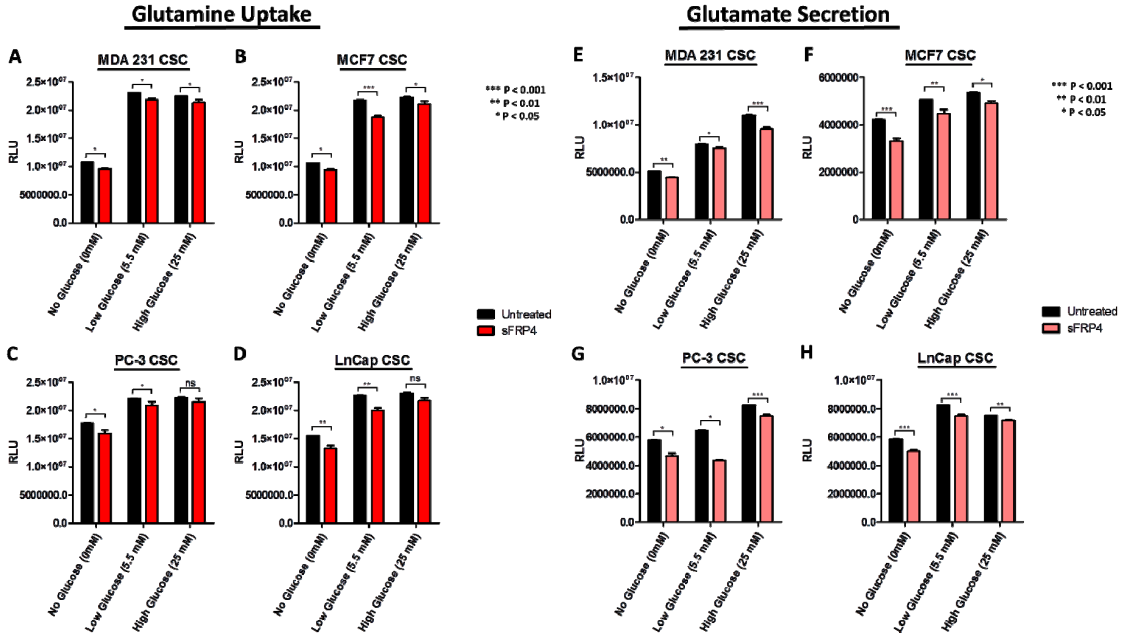

Figure 5. Extracellular metabolites with sFRP4 treatment of CSCs: Changes in glutamine uptake and glutamate secretion in CSCs grown in culture medium with increasing glucose concentrations were measured. (**A**,**E**) MDA231 CSCs; (**B**,**F**) MCF7 CSCs; (**C**,**G**) PC-3 CSCs; and (**D**,**H**) LnCap CSCs were treated with sFRP4 for 24 h. Statistical analysis was performed using ANOVA for analysis variance with Bonferroni test for comparison showing significance as *** $p < 0.001$; ** $p < 0.01$; * $p < 0.05$. Data are mean ± standard error of mean from three independent experiments.

2.6. Changes in Redox Signature with sFRP4 Treatment of CSCs

The nicotinamide adenine dinucleotide redox couple (NAD+/NADH) is a marker of catabolism. Here we aimed at describing the divergent redox profile of the CSC population isolated from breast and prostate tumour cell lines under different glucose concentrations. We also demonstrated the effect that sFRP4 has on the divergent redox signature of these CSCs (Figure 6). The results show the comparison of NAD+/NADH ratio in CSCs with no, low, and high glucose content, and the effect of sFRP4 in these conditions. The NAD+/NADH ratio was considerably higher in MDA231 CSCs, and sFRP4 treated CSCs showed a significant decrease (Figure 6A). The NAD+/NADH ratio in MCF-7 CSCs gradually decreased with an increase in glucose concentration, whereas sFRP4 had a minimal effect (Figure 6B). In PC3 CSCs, the NAD+/NAD ratio had an inverse activity as compared to MCF7 CSCs; here the ratio increased with an increase in glucose concentration, and sFRP4 treatment oscillated CSC catabolism by increasing the ratio in the no-glucose group, whereas it decreased the ratio in low and high-glucose groups (Figure 6C). The NAD+/NADH ratio in LnCap CSCs followed this trend, and decreased the redox activity as the glucose concentration increased; moreover, sFRP4 had no aberrant effect and decreased the ratio in two out of three glucose groups (Figure 6D). The generalised observation was that hormone-independent CSCs such as MDA231 (ER−) and PC3 (AR−) demonstrated a higher NAD+/NADH ratio with increasing glucose concentrations (Figure 6A,C); whereas hormone-dependent CSCs MCF7 (ER+) and LnCap (AR+) demonstrated a decrease in the NAD+/NADH ratio with an increase in glucose concentration (Figure 6B,D).

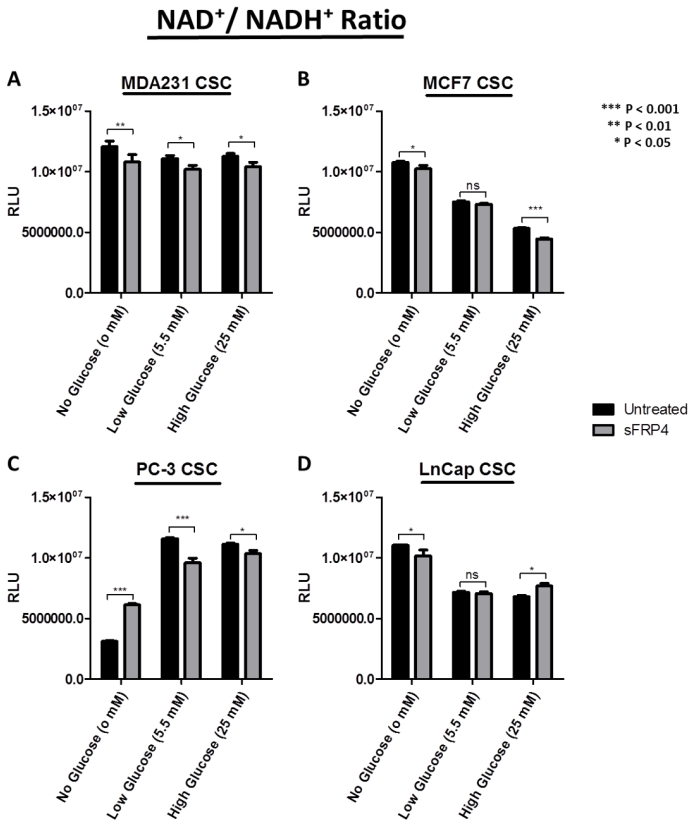

Figure 6. Redox signature with sFRP4 treatment of CSCs: Comparison of NAD+/NADH ratio in CSCs with no, low, and high glucose content. (**A**) MDA231 CSCs; (**B**) MCF7 CSCs; (**C**) PC-3 CSCs; and (**D**) LnCap CSCs were treated with sFRP4 for 24 h. Statistical analysis was performed using ANOVA for analysis variance with Bonferroni test for comparison showing significance as *** $p < 0.001$; ** $p < 0.01$; * $p < 0.05$. Data are mean ± standard error of mean from three independent experiments.

2.7. The Effect of sFRP4 on CSC Metabolism Target Proteins

Following CSC isolation in different glucose concentrations and sFRP4 treatment, we investigated the post-translational modifications in CSCs for a central regulator of cell metabolism (mTOR), AMP-activated protein kinase (AMPK), rate-limiting enzyme (acetyl-CoA carboxylase 2), metabolic oncogene (fatty acid synthase), metabolic gatekeeper (pyruvate dehydrogenase), glucose transporter (GLUT4), and Bcl-2 associated death promotor [19]. MTOR was highly expressed in all the untreated groups but decreased when treated with sFRP4 in low and high glucose groups, except for the LnCap CSC high glucose group (Figure 7D).

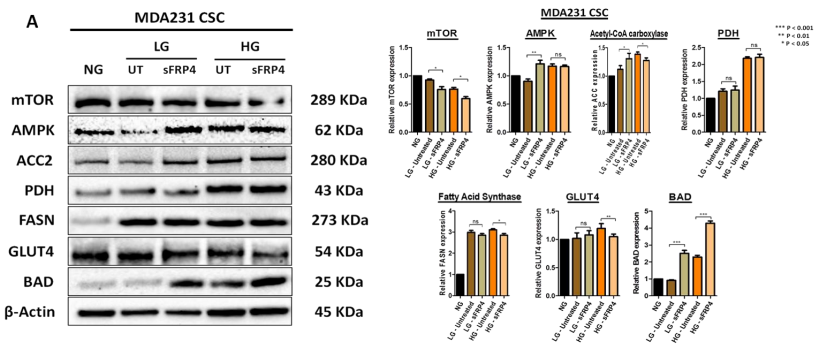

Figure 7. *Cont.*

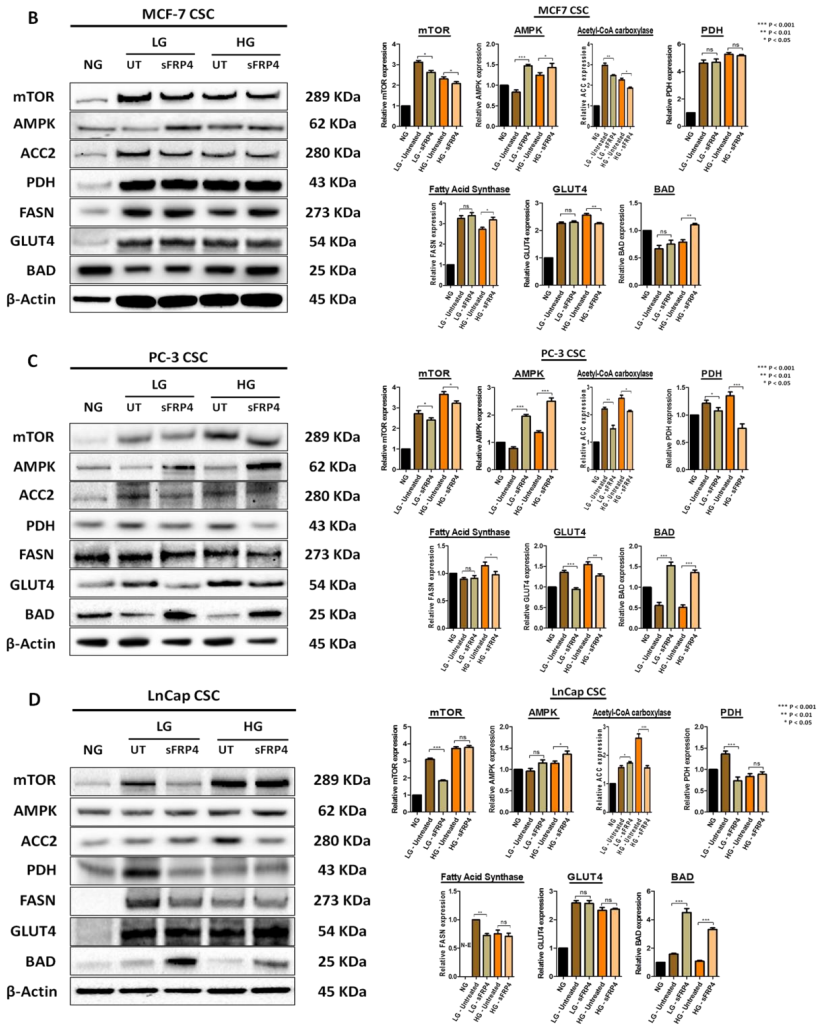

Figure 7. The effect of sFRP4 on CSC metabolism target proteins: Changes in CSC metabolic profile and the post-translational modifications with increasing glucose conditions. (**A**) MDA231 CSCs; (**B**) MCF7 CSCs; (**C**) PC-3 CSCs; and (**D**) LnCap CSCs were treated with sFRP4 for 24 h. Densitometry analysis was performed using ANOVA for analysis variance with Bonferroni test for comparison showing significance as *** $p < 0.001$; ** $p < 0.01$; * $p < 0.05$; *ns*—non-significant. Blots and relative protein expressions are mean ± standard error of mean from three independent experiments.

The AMPKα protein levels were observed in all the glucose groups and were significantly elevated with sFRP4 treatment, except the MDA231 CSC high glucose group (Figure 7A). This indicates the AMPKα activity in regulating mTOR, indicating the role of AMPK in the PI3K/AKT/mTOR signalling cascade. The protein levels of acetyl-CoA carboxylase 2 (ACC2), a main isoform in lipogenic and oxidative tissues, decreased in all CSC high glucose groups following sFRP4 treatment, whereas there was an increase post-sFRP4 treatment in the MDA231 CSC low glucose group (Figure 7A). Overexpression of pyruvate dehydrogenase confers higher pyruvate conversion to acetyl-CoA; however, sFRP4 treatment significantly decreased ACC2 protein expression in PC3 CSCs for all glucose groups (Figure 7C) and LnCap CSC low glucose groups (Figure 7D). There was a reduction in fatty acid synthase (FASN) protein expression following treatment with sFRP4 in high-glucose groups; however, there was a minimal effect in all the CSCs in low-glucose groups except LnCap CSCs. The glucose transporter GLUT4 protein expression in all CSCs increased with an increase in glucose concentration; however, GLUT4 decreased with sFRP4 treatment in all CSCs in the high glucose group, and only the PC3 CSCs low-glucose group exhibited a decrease in GLUT4 expression post-sFRP4

treatment (Figure 7C). Expression of Bcl-2 associated death promotor BAD was lower in untreated CSCs but increased significantly with sFRP4 treatment across all glucose groups. The increased BAD protein expression confirms the pro-apoptotic role of sFRP4.

3. Discussion

It has been established for decades that cancer cells actively use glycolytic metabolism even in the presence of oxygen, which is known as the Warburg effect [14,15]. The cancer cells are benefited by the high glycolytic rate for ATP production and other metabolites [20]. Due to this prevalent glycolytic elevation in cancer cells and its clinical relevance, such metabolic alterations have been considered as the hallmark of cancers [21]. Recent studies suggest that CSCs may have even more active glycolytic activity compared to the bulk of tumour cells [11,13,22]. Consistently, the current study showed the CSCs to be highly glycolytic, and these observations underscore the importance of glucose as an essential nutrient for CSCs and suggest the possibility that levels of glucose in the tumour microenvironment might significantly affect CSC survival. Therefore, the knowledge of CSC metabolism is of great importance for our understanding of reproductive tumours (i.e., breast and prostate), which are tumours with poor prognosis. To gain further insights into CSC metabolism, we investigated the role of sFRP4 on CSCs isolated in culture medium with various glucose concentrations. Our study demonstrates sFRP4 elicits an anti-proliferative effect and causes spheroid disruption, and decreases glucose uptake, glutamine uptake, glutamate secretion, redox signature, and the signalling cascade responsible for cell survival, and also promotes apoptosis within the CSCs, therefore indicating sFRP4's potential role in regulating CSC metabolism.

While the important role of tumour tissue niches in affecting CSCs has gained attention in recent years, the impact of key nutrients in the tumour microenvironment remains largely unknown. The CSC niches preserve the CSCs' phenotypic plasticity, facilitate metastatic potential, and support high expression of drug efflux transporters, making them highly chemo-resistant [23]. In this study, we used various functional assays to evaluate the effect of glucose and sFRP4 on CSCs, and used Western blot analyses to investigate the underlying mechanisms. Our results exemplify that glucose plays a major role in promoting the CSC phenotype (Figure 1). We also showed that targeting the Wnt signalling pathway by using sFRP4 has the capacity to disrupt the CSC niches in various glucose concentrations (Figure 1). CSCs in a glucose depleted medium were morphologically stressed and more susceptible to sFRP4. Spheroid disruption by sFRP4 decreases the CSCs' plasticity and cell-cell adhesion, initiating the CSCs' differentiation towards tumour cells and reducing their self-renewal capacity. The induction of CSCs by glucose appears to be a reversible phenomenon. As shown in Figures 1 and 2, switching CSCs from a no glucose medium to a low glucose or high glucose medium led to an increase in their viability and they became morphologically more robust. Consistently, glucose deprivation caused rapid depletion in CSC viability, which re-appeared when glucose was replenished.

In previous studies, our group has shown that sFRP4 has an anti-proliferative capacity in CSCs derived from breast, prostate, ovary, glioblastoma multiforme, and head and neck tumours [8,24,25]. In this study we demonstrated that sFRP4 decreased the viability of CSCs in increasing glucose concentrations when compared to CSCs in a glucose depleted medium (Figure 2), indicating sFRP4s' anti-proliferative capacity is independent of exogenous key nutrients in the microenvironment.

Most cancer cells rely more on glycolysis rather than on oxidative phosphorylation for glucose metabolism [14]. The active utilization of glucose by tumour cells constitutes the basis of 2-[^{18}F]fluoro-2-deoxy-D-glucose positron emission tomography (^{18}FDG-PET) imaging for cancer diagnosis, and positive FDG-PET signals post-treatment predict poor prognosis [26–29]. CSCs are chemo-resistant cells, and it is possible that with conventional chemotherapeutic treatment the residual lesions within the tumour would enrich for CSCs with elevated glycolytic activity. In a previous study, CSCs were isolated from human non-small cell lung carcinoma (NSCLC) and colon cancer cell lines [30]. Using flow cytometry, they compared the glucose metabolic activity between CSCs and non-CSCs; glucose uptake was significantly increased in CSCs compared to non-CSCs, indicating

that CSCs were more glycolytic than their normal counterparts. To better understand the effect of glucose on glycolytic metabolism, we isolated CSCs in glucose deprived medium and increasing concentrations of glucose. We observed high glycolytic activity within CSCs isolated in low glucose (5.5 mM) medium, and comparatively less in high glucose (25 mM) medium. Meanwhile, we also investigated the effect of sFRP4 on glucose uptake, where we observed varying effects. We postulate that sFRP4 had stressed the CSCs in the glucose deprived medium, driving them to initiate higher glycolytic activity; whereas CSCs in high glucose medium showed an inhibitory effect on glucose uptake, suggesting that sFRP4 has a direct effect on glycolytic flux. Furthermore, the glucose uptake was variable in all CSCs, and no trend was observed. Other studies have also found that there is no differential response to epidermal growth factor [31] and basic fibroblast growth factor (FGF) in the media used to isolate CSCs, ruling out any interference of these growth factors on CSC glucose uptake [32]. Although the presence of glucose in CSC culture medium has been shown to significantly increase the viability of CSCs in the overall isolation process, glucose uptake is an essential process and a key nutrient for CSCs [30]. A recent proteomic and targeted metabolomics analysis between breast CSCs and their counterpart revealed a metabolic pathway associated with the stem-like conditions, indicating that breast CSCs shift from mitochondrial OXPHOS towards fermentative glycolysis [33].

Although cancer cells exhibit high rates of glycolysis, their mitochondrial OXPHOS remains intact and becomes progressively more dependent on glutamine metabolism [34]. In cancer cells, the rate of glutamine conversion to lactate is higher compared to normal cells, which represents an alternative metabolic pathway to glucose consumption in a glucose depleted microenvironment [35]. Glutaminase converts glutamine to glutamate through glutaminolysis. Glutaminase and glutamine levels in the cell culture medium correlate with the cancer cell proliferation, whereas glutamate levels are associated with tumour aggressiveness [36]. Moreover, glutamine's function to promote cell growth is widely dependent on the epigenetic background of the tumour [37,38]. Glutamine depletion induces apoptosis in melanoma and prostate cancer cells, and using acivicin to inhibit glutaminolysis has been very effective in animal models of cancer [39–41]. In addition, another study demonstrated the targeting of glutamine uptake as a new therapeutic strategy to treat acute myeloid leukaemia [42]. However, glutamine deprivation of CSCs is less well characterized. Morover, previous evidence has suggested that intra-mitochondrial protein AIF translocates to the nucleus and promotes caspase-independent cell death induced by glutamate toxicity [43]. We demonstrated in our study that sFRP4 significantly decreased the glutamine uptake and glutamate secretion in all CSC glucose groups, indicating the key role of sFRP4 in glucose/glutamine metabolism. Another study demonstrated that glutamine promotes cell growth in ovarian cancer cells by activating the mTOR/S6 and MAPK pathways [44]. This prompts us to suggest that targeting glutaminolysis by sFRP4 might prove a valuable step in regulating CSC metabolism.

The NAD+/NADH ratio is directly impacted by glycolytic and mitochondrial activities that change during metabolic reprogramming. The NAD+/NADH redox state plays a key role in cancer cell stemness [45]. Nicotinamide, the NAD precursor, protects cells from apoptosis and senescence by accelerating cell proliferation and alleviating oxidative stress. Accordingly, in CSCs, increased glucose metabolism reduces the level of reactive oxygen species (ROS) to promote EMT [10,46], whereas the level of NADH is decreased with a decrease in the ratio of reduced glutathione (GSH) to oxidised (GSSH) glutathione [47]. A high NADH level is a property that is conserved between normal and cancerous stem cells [48]. A previous study has also demonstrated that when CSCs are fed with mitochondrial fuel (L-lactate or ketone bodies), CSCs quantitatively produce more NADH in response to the stimulus compared to non-CSCs [49]. In addition, NAD+ depletion, using the NAMPT inhibitor FK866, potently blocked spheroid formation [48]. We demonstrated a significant reduction in the NAD+/NADH ratio in all CSCs post-sFRP4 treatment, suggesting that a higher NAD+ content is important for enhancing the resistance to stress induced by ROS in CSCs; whereas a decreased NAD+/NADH ratio makes CSCs more susceptible to reprogramming their redox state.

There is growing evidence on the role of the mTOR pathway in the maintenance of CSCs. Prostate cancer radio-resistance is associated with EMT and enhanced CSC phenotypes via activation of the PI3K/Akt/mTOR signalling cascade [50]. Activation of the mTOR signalling pathway enhances breast CSC colony formation ability in vitro and tumorigenicity in vivo [51]. Suppression of mTOR decreases ALDH1 activity in colorectal CSCs [52,53]. In glioblastoma CSCs, cross-inhibitory regulation between the MEK/ERK and PI3K/mTOR signalling cascades contributed to self-renewal and tumorigenic capacity [54]. Aberrant activation of the PI3K/Akt/mTOR signalling pathway leads to an increase in chemokine (C-X-C motif) receptor 4 (CXCR4), which corresponds to maintenance of stemness in NSCLC cells [55]. Interestingly, metformin decreased radio-resistance of CSCs in mouse fibrosarcoma cells and human MCF7 breast cancer cells by activating AMP-activated protein kinase and suppressing mTOR expression [56]. We demonstrated that sFRP4 decreases mTOR protein expression and increases AMP kinase (AMPK) expression, which may inhibit the PI3K/Akt/mTOR signalling cascade via phosphorylation of mTOR. It is possible that the anti-tumour activity of sFRP4 in vitro maybe associated with inhibition of the insulin/IGF-1 pathway through AMPK activation. AMPK regulates mTOR activity through activation of the tuberous sclerosis protein 1/2 complex [57,58].

In addition to glucose and glutamine, fatty acids are an important energy source incorporated in extracellular media, or can be obtained endogenously by accumulating lipid droplets [59]. Fatty acid synthesis is an anabolic process, which starts with converting acetyl CoA to malonyl CoA by acetyl CoA carboxylase. We found a higher expression of fatty acid synthase (FASN) in breast CSCs for all glucose groups. Notably, a high expression of FASN has been linked to poor prognosis of pancreatic ductal adenocarcinoma patients and depends heavily on induction of EGFR/ERK signalling [60]. Furthermore, FASN promotes EMT in ovarian [61], breast [62], and colorectal [63] cancers. Inhibition of FASN leads cancer cells to apoptosis, mainly by inhibiting DNA replication and the production of anti-apoptotic proteins [64]. We saw a minimal effect of sFRP4 on FASN, although breast CSCs in the high glucose groups responded with a decrease in FASN, and the LnCap CSC low glucose group had a maximal decrease in FASN. We propose that sFRP4's effect is associated with the glycolytic switch of CSCs occurring in different glucose concentrations.

Pyruvate dehydrogenase (PDH) is a key enzyme that mediates the entry of pyruvate to mitochondria where it facilitates its conversion to acetyl CoA. PDH activity is regulated by pyruvate dehydrogenase kinase 1 (PDK-1) [65]. In CSCs, PDK-1 via the TCA cycle, phosphorylates pyruvate dehydrogenase and suppresses the pyruvate to acetyl-CoA conversion. Furthermore, suppressing the metabolic flow of pyruvate in mitochondria induces the conversion of pyruvate to lactate in the cytosol [30,66]. We observed a decrease in Acetyl-CoA Carboxylase (ACC2) expression in CSCs after treatment with sFRP4, indicating the inhibitory effect of the predominant isoform in lipogenic and oxidative tissues and the mitochondrial membrane potential. Higher PDH expression was observed in CSCs in the high glucose group, and sFRP4 had an impairing effect on PDH expression in prostate CSCs. PC-3 CSC treatment with sFRP4 decreased PDH expression in both glucose groups. A recent study revealed that chemical inhibition (via soraphen A) of acetyl CoA carboxylase suppresses self-renewal growth of CSCs derived from the MCF7 cell line [67]. However, the conversion of pyruvate to acetyl CoA in CSCs is still unclear, and how glucose concentration might influence the process is still something worth exploring.

Tumorigenesis is associated with enhanced cellular glucose uptake and increased metabolism. The transmembrane glucose transporter (GLUT) proteins mediate glucose uptake in cancer cells, and initiate the glucose utilisation cascade [68]. GLUT4 is aberrantly expressed in many tumours, though no study has been undertaken within CSCs. We demonstrated that GLUT4 protein levels are increased in all CSCs, and there is increased expression with an increase in glucose concentration. Furthermore, we also observed the inhibitory effect of sFRP4 on GLUT4 protein expression in prostate CSCs.

Most anti-cancer drugs exert their effect through triggering the apoptosis pathway, although CSCs escape apoptosis by altering their expression levels of pro-apoptotic and anti-apoptotic Bcl-2

family members [8]. BAD (Bcl-2 associated death promoter) is a member of the Bcl-2 family that, when dephosphorylated, initiates apoptosis by heterodimerizing with anti-apoptotic proteins Bcl-xl and Bcl-2 (15). In vivo, BAD phosphorylation was detected in CSCs of 83% breast cancer biopsies [69]. The overexpression of BAD is correlated with chemo-resistance. Interestingly, high-grade tumours exhibit higher BAD protein levels than those with low-grade cancer, suggesting a role in tumour progression [70]. We demonstrated a gradual increase in (dephosphorylated) BAD expression in CSCs treated with sFRP4. Moreover, sFRP4 treatment elevated BAD in all glucose groups. The increased expression of dephosphorylated BAD is an indicator for apoptosis, and an increased expression depicts the activation of caspase cleavage [71]. BAD expression was consistently high in all CSCs treated with sFRP4, and the elevated expression of apoptotic proteins within all the CSCs reinforces sFRP4's role as a pro-apoptotic agent.

4. Materials and Methods

4.1. Cell Culture

Monolayer Cell Culture

Cell culture plates for adherent cells were purchased from Nunc^{TM} (ThermoFisher Scientific, Waltham, MA, USA). The human breast tumour cell lines MDA-MB 231 (ER−) and MCF-7 (ER+), and human prostate tumour cell lines PC-3 (AR−/PSA−) and LnCap (AR+) were purchased from American Type Culture Collection (ATCC, Manassas, VA, USA). The cells were cultured in RPMI-1640 medium (#11875-093, Gibco, ThermoFisher Scientific) supplemented with 10% foetal bovine serum (#SFBS, Bovogen, Victoria, Australia) and 100 U/mL PenStrep (#15070063, Life Technologies, Carlsbad, CA, USA). All cells were maintained at 37 °C in a humid incubator with 5% CO_2.

4.2. Cancer Stem Cell Isolation

For CSC isolation, culture plates with an ultra-low-attachment surface were purchased from Corning Life Sciences (Corning, NY, USA). CSCs were cultured in serum-free medium (SFM) containing DMEM-No Glucose (Gibco, US #11966025), DMEM-Low Glucose, 5.5 mM (#SH30021.01, HyClone, South Logan, UT, USA), and DMEM-High Glucose, 25 mM (#SH30081.02, HyClone) supplemented with the growth factors bFGF (20 ng/mL) (#cyt-085, ProSpec Bio, Rehovot, Israel), EGF (20 ng/mL, #cyt-217, ProSpec Bio), and 1× B27 (#17504044, Gibco), and 100 U/mL PenStrep (#15070063, Life Technologies). CSC-enriched populations of cells were obtained by plating a single cell suspension of breast and prostate tumour cells at 10,000 cells/cm^2 in SFM on Low-adherent six-well plates (#3471, Corning). CSCs were isolated in SFM; the spheroids were formed at the 3rd day of plating tumour cells. To analyse the effects of sFRP4, cells were cultured in medium supplemented with sFRP4 (see Section 4.3 for details).

4.3. CSC Treatment

The CSCs were treated in this study with purified sFRP4 (#1827-SF-025, R&D Systems, Minneapolis, MN, USA). CSC sensitization with sFRP4 was performed by adding sFRP4 to the cell culture at 250 pg/mL [8] for 24 h at 37 °C in a 5% CO_2 incubator.

4.4. Viability Assay

A cell counting viability kit (CCK8, #96992, Sigma-Aldrich, St. Louis, MO, USA) was used for the quantitation of viable cells. Monolayer cells were plated with culture medium varying in glucose content at a density of 10,000 cells/cm^2 in a low-adherent flat-bottomed 96-well plate (Corning #3474) for 3 days in non-adherent SFM conditions. Wells with treatment-free medium were used as a negative control. CSCs were treated with sFRP4 for 24 h, then 10 µL of CCK8 solution was added to each well

and incubated at 37 °C in a 5% CO_2 incubator for 1 h. Plates were read at 450 nm using an EnSpire Multilabel Plate Reader (Perkin-Elmer, Waltham, MA, USA).

4.5. Cell Surface Markers

To assist in determining their identity, cell surface markers were examined in CSCs by flow cytometry (BD FACSCANTO II, BD Biosciences, San Jose, CA, USA) using CellQuest data acquisition and analysis software. APC-CD44 (1:100) (#338805, BioLegend, San Diego, CA, USA), PE Cy7-CD24 (1:10) (#311119, BioLegend), and PE-CD133 (1:100) (#372803, BioLegend). Cells incubated with conjugated irrelevant IgGs were used as negative controls. Tumour specific CSC markers used were: breast CSCs ($CD44^+/CD24^{-/low}$) [4,72] and prostate CSCs ($CD133^+/CD44^+$) [73]. CSCs were characterized by flow cytometry (BD FACSCANTO II), as previously published [8]. These data are not shown since the surface markers were used only to characterize CSCs.

4.6. The Cancer Genome Atlas Dataset

To analyse the relationship between *sFRP4* and *AMPKB1* (AMP Kinase), *mTOR* (mammalian target of rapamycin), *GLUT1* (glucose transporter), *SLC1A5* (glutamine transporter), *BAD* (Bcl-2 associated death promotor), and *PDHA1* (pyruvate dehydrogenase) in breast and prostate cancers, we obtained data from TCGA, Nature 2011 by using www.cbioportal.org [74,75]. On the home page of the website, select 'download data', then select for breast "Breast Invasive Carcinoma (TCGA, Nature 2012)", and for prostate "Prostate Adenocarcinoma (TCGA, Cell 2015)", click "mRNA expression Z-score (all genes)" from select genomic profiles and enter gene set for e.g.,: "sFRP4 AMPKB1", select "Transpose data matrix" and click submit. The sFRP4 and *AMPKB1* (encoding AMPK) mRNA Z-scores for 825 cases (Breast) and 333 cases (Prostate) will appear. The same process was followed for all the genes examined. The correlation between these Z-scores of two genes was then analyzed by Spearman correlation and Pearson correlation, and plotted using GraphPad Prism V5.0 (GraphPad software, La Jolla, San Diego, CA, USA).

4.7. Glucose Uptake in CSCs

The bioluminescent glucose uptake assay was applied to CSCs in 96-well low adherent white luminescent plates. Before beginning the assay, the culture medium was removed and the CSCs were washed with 100 µL of phosphate-buffered saline (PBS). To initiate glucose uptake, 50 µL of 2-Deoxy-D-Glucose (2DG) (1 mM) in PBS was added to cells. The uptake reaction was stopped and samples were processed as described in the standard protocol of the Glucose Uptake Glo Assay kit (#J1342, Promega, Madison, WI, USA). Because glucose uptake is time dependent, the optimal assay time was determined by stopping the reaction at the 90 min point. This is the time frame that was chosen for standard glucose uptake conditions in CSCs. The luminescent signal produced by this assay is proportional to the rate of glucose uptake, but the precise rate of glucose uptake can be calculated by taking into account the number of cells (10,000 cells/well), time of uptake (90 min), and the amount of 2-Deoxy-D-Glucose-6-phosphate (2DG6P) produced (µM), as measured using the standard protocol. The rate of glucose uptake was measured as fmol/min/cell [76]. Luminescence was read with 0.3–1 s integration on a luminometer (EnSpire Multilabel Plate Reader, Perkin-Elmer).

4.8. Detection of Extracellular Metabolites in CSC Medium

For measuring changes in glutamine and glutamate in the CSC medium, CSCs were isolated from MDA231, MCF-7, PC-3, and LnCap cells and plated in SFM conditions in low adherent 96-well plates at a density of 10,000 cells/cm^2. CSCs were grown in 100 µL DMEM medium supplemented with variable glucose concentrations, 4 mM glutamine, and growth factors (see Section 4.2, CSC isolation). The cells were incubated in a tissue culture incubator (37 °C, 5% CO_2), and were treated with sFRP4 (250 pg) on the 3rd day for 24 h. At the indicated time (i.e., 24 h treatment), 2 µL of culture medium was removed and transferred to a separate 96 well-plate containing 98 µL PBS/well. For metabolite analysis,

4.5 µL of thawed sample was transferred to respective 96 well white luminescent plates for glutamine and glutamate detection. Samples were then assayed as described in Glutamine/Glutamate-Glo Assay (#J8021, Promega) standard protocols [77]. Luminescence was read on a luminometer (EnSpire Multilabel Plate Reader, Perkin-Elmer).

4.9. Detection of Qualitative NAD+/NADH in CSCs

The NAD+/NADH ratio was quantified by a luciferase assay provided in the NAD+/NADH Glo Assay kit (#G9071, Promega). CSCs were isolated from MDA231, MCF-7, PC-3, and LnCap cells plated in SFM conditions in low adherent 96-well plates at a density of 10,000 cells/cm^2. The cells were incubated in a tissue culture incubator (37 °C, 5% CO_2), and were treated with sFRP4 (250 pg) on the 3rd day for 24 h. Briefly, after appropriate treatment over the desired time, the medium was removed and cells were supplemented with 50 µL of PBS and 50 µL of 0.2 N NaOH solution with 1% DTAB to obtain a cell lysate. To measure NAD+, a 50 µL aliquot of cell lysate was treated with 0.4 N HCL and heat quenched at 60 °C for 15 min. The solution was neutralized with Trizma buffer. NADH samples were heat quenched following the addition of NaOH with 1% DTAB and the solution was neutralized with HCL-Trizma. An equal volume of NAD/NADH-Glo Detection Reagent was added to each well with cell lysate, incubated at room temperature for 60 min, and Luminescence was read on a luminometer (EnSpire Multilabel Plate Reader, Perkin-Elmer).

4.10. Western Blotting

CSCs were washed twice with PBS and then lysed in RIPA lysis buffer (#R0278, Sigma) (150 mM NaCl, 1.0% IGEPAL® CA-630, 0.5% sodium deoxycholate, 0.1% SDS, 50 mM Tris pH 8.0, and Proteinase Inhibitor 1×). Post sonication, cell lysates were centrifuged at 14,000 *g* for 10 min at 4 °C, and the supernatants were used for western blotting. The lysates were resolved by sodium dodecyl sulphate-polyacrylamide gel electrophoresis, transferred onto nitrocellulose membranes, and then stained with 0.1% Ponceau S solution (#P3504, Sigma) to ensure equal loading of the samples. After being blocked with 5% non-fat milk for 60 min, the membranes were incubated with primary antibodies mTOR (7C10) (1:1000, #2983, Cell Signaling, Danvers, MA, USA); AMPKα (1:1000, #2532, Cell Signaling); acetyl-CoA carboxylase (C83B10) (1:1000, #3676, Cell Signaling); fatty acid synthase (C20G5) (1:1000, #3180, Cell Signaling); pyruvate dehydrogenase (C54G1) (1:1000, #3205, Cell Signaling); GLUT4 (1:2500, #ab65267, Abcam, Cambridge, UK); BAD (1:500, #sc-8044, SantaCruz, TX, USA); and β-Actin (13E5) (1:1000, #4970, Cell Signaling) overnight at 4 °C, and the bound antibodies were visualized with horseradish peroxidase-conjugated secondary antibodies using the ECL Western Blotting Substrate (GE #RPN2106, Amersham, Pittsburgh, PA, USA) on a Chemi-Doc (Bio-Rad, Hercules, CA, USA) imaging analyser.

5. Conclusions

In summary, sFRP4 plays an important role in breast and prostate CSC metabolism by reducing the CSCs' proliferative capacity and glucose uptake, modulating their redox signature, and decreasing the CSCs' survival signalling cascade by targeting the mTOR complex, making them more responsive to therapy. Further in vivo studies may confirm the efficacy of sFRP4 in altering CSC metabolism to prevent tumour relapse and lead to tumour resolution.

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