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CANCER STEM- LIKE CELLS FROM HEAD AND NECK CANCERS ARE CHEMOSENSITIZED BY THE WNT ANTAGONIST, sFRP4, BY INDUCING APOPTOSIS, DECREASING STEMNESS, DRUG RESISTANCE, AND EPITHELIAL TO MESENCHYMAL TRANSITION

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Cancer stem cells (CSCs) of head and neck squamous cell carcinoma (HNSCC) are defined by high self-renewal and drug refractory potential. Involvement of Wnt/β-catenin signaling has been implicated in rapidly cycling cells such as CSCs, and inhibition of the Wnt/β-catenin pathway is a novel approach to target CSCs from HNSCC. In this study, we found that an antagonist of FrzB/Wnt, the secreted frizzled-related protein 4 (sFRP4), inhibited the growth of CSCs from two HNSCC cell lines, Hep2 and KB. We enriched the $CD44^+$ CSC population, and grew them in spheroid cultures. sFRP4 decreased the proliferation and increased the sensitivity of spheroids to commonly used drug in HNSCC, namely cisplatin. Self-renewal in sphere formation assays decreased upon sFRP4 treatment, and the effect was reverted by the addition of Wnt3a. sFRP4 treatment of spheroids also decreased β-catenin, confirming its action through the Wnt/β-catenin signaling pathway. Quantitative PCR demonstrated a clear decrease of the stemness markers CD44 and ALDH, and an increase in CD24 and drug resistance markers ABCG2 and ABCC4. Furthermore, we found that after sFRP4 treatment, there was a reversal in the expression of

epithelial to mesenchymal (EMT) markers with the restoration of the epithelial marker E-cadherin, and depletion of EMT-specific markers twist, snail, and N-cadherin. This is the first report demonstrating that the naturally occurring Wnt inhibitor, sFRP4, can be a potential drug to destroy CSC-enriched spheroids from HNSCC. The repression of EMT and the decrease in stemness profile further strengthen the use of sFRP4 as a potent therapeutic against CSCs.

Key words- Head and neck cancer, cancer stem cells, sFRP4, Wnt signaling, apoptosis, EMT

1.1 INTRODUCTION

Head and neck squamous cell carcinoma (HNSCC) is the sixth most prevalent cancer worldwide (Van der Geer et al., 2010). Mortality from HNSCC is high because of distant metastases and the emergence of therapy-resistant tumors. Prognosis of HNSCC is determined by the degree of lymphatic invasion and metastasis at diagnosis (Parkin et al., 2005). The standard of care for patients with HNSCC includes primarily platinum-based chemotherapeutic drugs, surgery, and radiotherapy (Forastiere et al., 2001). Despite this, the 5-year survival rate for these patients has still been in the range of 50–60% for the last 3 decades (Carvalho et al., 2005).

Cancer stem cells (CSCs), a small population of cells in most tumors, support tumor growth through their ability to self-renew and differentiate into heterogeneous tumor tissue. CSCs are typically resistant to radiation therapy and standard cytotoxic agents, which results in their enrichment and in the consequent recurrence of refractory tumors. After initially being identified in leukemia (Bonnet and Dick, 1997), they have been found in many solid tumors. CSCs have been identified in human HNSCCs using markers such as CD133 and CD44, and by aldehyde dehydrogenase (ALDH) activity (Prince, 2007). The presence of CSCs in HNSCC has been reported in many studies and has been comprehensively reviewed by Minnelli and Gallo (Mannelli and Gallo, 2012). The HNSCC CSCs reside in perivascular niches in the invasive front, where endothelial cells contribute to their survival and function.

Targeting key signaling pathways that are active in CSC self-renewal is one approach to cancer therapy. Abnormal activation of the Wnt/β-catenin signaling pathway has been described in a wide variety of human cancers and in CSCs, and has already been demonstrated in HNSCC (Lim

et al., 2012). Wnt dysregulation has a role in inducing epithelial to mesenchymal transition (EMT) transcription factors Twist and Snail (Yang et al., 2006, Reya and Clevers, 2005, Song et al., 2010). CSCs from hepatic carcinoma cell lines exhibited enhanced chemoresistance to cisplatin, which was reversed by lentiviral microRNA knockdown of β-catenin (Yang et al., 2008). Similar studies have demonstrated that the Wnt/β-catenin signaling pathway was also able to confer chemoresistance to 5- fluorouracil and doxorubicin (Noda et al., 2009, Flahaut et al., 2009). Activation of the Wnt pathway, by the Wnt receptor frizzled-1, induced expression of the multiple drug resistance gene *MDR1* and promoted resistance to doxorubicin in chemoresistant neuroblastoma cells (Flahaut et al., 2009). In ovarian CSCs, *ABCG2* was shown to mediate cisplatin and paclitaxel resistance, which was reversed by β-catenin siRNA knockdown (Chau et al., 2012).

In this study, we investigated the effect of the naturally occurring protein secreted frizzled-related protein 4 (sFRP4), which is an inhibitor of Wnt signaling, on CSC-enriched spheroid cells from two HNSCC cell lines. We examined the ability of sFRP4 to chemosensitize spheroids to commonly used therapeutic drugs and studied CSC-specific properties such as self-renewal, the expression of CD44 positive cells, and the ability to form spheres in culture. To explore the underlying molecular mechanism of sFRP4, we examined the Wnt/ß-catenin signaling pathway, changes in the EMT profile with relation to sFRP4, and the expression of drug resistance markers. We identified that sFRP4 enhanced the chemosensitivity of CSC-enriched cells by inducing apoptosis, reducing EMT, and decreasing stemness properties. Our findings are relevant in the context of designing drugs targeting CSCs, as sFRP4 is a natural Wnt antagonist acting only on cells having dysregulated Wnt/ß-catenin signaling. These studies identify sFRP4 as a key Wnt antagonist that can regulate CSC maintenance and chemoresistance, which can lead to better targeted modes of cancer therapy.

1.2 MATERIALS AND METHODS

1.2.1 CELL LINES

The two cell lines Hep2 (laryngeal carcinoma) and KB (tongue carcinoma) used in this study were purchased from the National Center of Cell Sciences, Pune, India, and cultured and maintained in DMEM/F-12 and DMEM-LG (Gibco) (1:1) containing 1X GlutaMax (Life

Technologies), 10% Fetal Bovine Serum (Hyclone), and 100U/mL PenStrep (Life Technologies). Normal non-tumorigenic mouse embryonic fibroblasts (MEFs), were isolated from mouse embryo day e10.5 and cultured the DMEM-KO medium containing 1X GlutaMax (Life Technologies), 10% Fetal Bovine Serum (Hyclone), and 100U/mL PenStrep (Life Technologies). Serum free medium (SFM) for sphere culture was performed in basal medium (DMEM/F-12 + DMEM-LG) with 100U/mL PenStrep, 2mM GlutaMAX, and containing 20 ng/mL each of epidermal growth factor (EGF), basic fibroblast growth factor (bFGF; R&D Systems), and leukemia inhibitory factor (LIF; Chemicon). All cells were cultured at 37°C in a humid incubator with 5% CO_2. CSC populations were enriched by plating a single cell suspension of Hep2 and KB cells at 5,000 cells per mL in SFM on non-adherent 6 well plates (BD Biosciences) as reported previously (Warrier et al., 2014). The medium was replenished once in 2 days by centrifuging for 3 min at 450 × *g* at room temperature, aspirating the medium, and replacing it with fresh stem cell medium till the spheres were >120 μm in size, as observed using an inverted phase contrast microscope (Nikon Eclipse TE 2000-S, Japan). Spheres were then characterized for the expression of CSC markers CD44 and ALDH by flow cytometry and qPCR. After identifying for CSC enrichment of the spheres, spheroids were used for further analysis.

1.2.2 PROLIFERATION ASSAYS

MTT

The TACS MTT assay kit (R&D Systems) was used according to the manufacturer's protocol to measure cell metabolic viability.

Hep2 and KB spheroids were cultured in non-adherent SFM conditions (10,000 cells/mL) in 96-well plates. Drugs used were sFRP4 (250pg/ml), cisplatin (10mM), and doxorubicin (50ug/mL). The following treatment groups were examined: sFRP4- (S), cisplatin- (C), sFRP4 + cisplatin (S+C), doxorubicin- (D), and sFRP4 + doxorubicin (S+D) for a treatment time of 24h. After drug treatment, an MTT assay was performed and the plates were read at 595 nm using a Victor 3 Multilabel Plate Reader (Perkin-Elmer). As the combination of S+C was the most effective, subsequent analysis was performed using C and S+C.

BrdU ASSAY

Hep2 and KB spheroid cultures were subjected to the following drug conditions: sFRP4-250pg/mL (S), cisplatin- 10mM (C), and a combination of sFRP4 and cisplatin (S+C). The same drug treatment was performed for the remainder of the experiments. Hep2 and KB spheres were treated with various drugs, and cell proliferation was measured using the BrdU Cell Proliferation Assay Kit (Cell Signalling Tech Inc) according to the manufacturer's protocol. Plates were read at 450nm using a Victor 3 Multilabel Plate Reader.

1.2.3 SECONDARY SPHERE FORMING ASSAY

Primary spheres were obtained by plating 10,000 cells/mL, under SFM conditions, in ultra-low adherence, 6-well culture plates. On day 2, primary spheres were subjected to drug treatment as indicated. After treatment, the primary spheres from each condition were disrupted into single cell suspensions and were replated for secondary sphere formation under each condition. The secondary spheres were observed for morphology, and the total number of spheres were counted, together with the number of spheres of different sizes using a bright field phase contrast microscope (Nikon- Eclipse TE 2000-S), and photographs were taken using Qimaging- QICAM-fast 1394.

1.2.4 CASPASE ASSAY

After treatment of Hep2-spheroids and KB-spheroids with the various drug treatments, the activity of apoptosis-inducing caspase-3 was assayed using the EnzChek DEVD-CHO Caspase3 Assay Kit (Invitrogen), which provides a fluorescent substrate R110. Intensity of the fluorescence is correlated with an increase in caspase-3 activity, and the fluorescence intensity was measured using a Victor 3 Multilabel Plate Reader at 485/535 nm (Ex/Em).

1.2.5 SEMI-QUANTITATIVE REVERSE TRANSCRIPTION-PCR:

Total RNA was extracted from Hep2-spheroids and KB-spheroids after drug treatment using the RNeasy Plus Mini kit (Qiagen) according to the manufacturer's instructions. Total RNA was reverse-transcribed using SuperScript III First-Strand Synthesis System (Invitrogen, Carlsbad, CA). RNA (1 μg) was mixed with 1 μL Oligo dT (50 μM) and 1 μL of dNTP (10mM), made up to 13 μL with DEPC treated water, and heated at 65°C for 10 minutes, followed by incubation on ice. After primer hybridisation, 7 μL reaction volume containing 5X first strand buffer, RNase

OUT (40U/μL), 0.1 M DTT, and Superscript III were added to the RNA and subjected to thermocycling (25°C, 5 min; 50°C, 60 min; 70°C, 15 min) in a Veriti 96 well thermal cycler (Applied Biosystems). Qualitative expression of markers for CSC, apoptosis, drug resistance, and EMT (primers from Sigma, sequence as indicated in Tables 1 and 2) were analyzed by PCR (95°C 30s; annealing temperature, 30s; 72°C 30s for 40 cycles) in a Veriti 96 well thermal cycler. Products were resolved in 1.5 % agarose gel electrophoresis and detected using ethidium bromide. Integrated density values (IDV) were calculated using Alpha manager.

1.2.6 REAL-TIME QUANTITATIVE REVERSE TRANSCRIPTION-PCR.

The mRNA expression of different genes obtained qualitatively was further quantified using iQ SYBR Green Supermix (Bio-Rad, Hercules, CA) in a real time PCR system. cDNAs and gene-specific primers were mixed with 2X iQ SYBR Green Supermix (Bio-Rad), and dispensed on a MicroAmp® Optical *8-Tube Strip.* Fluorescence shift was observed using a 7500 Real-time PCR system (Applied Biosystems). Reaction parameters were 50°C for 2 minutes, 95°C for 10 minutes, followed by 40 cycles of 95°C for 15 seconds and 60°C for 1 minute. PCR products were verified by melting curves. The relative abundance of mRNAs was obtained using the comparative cycle threshold method and was normalized to the housekeeping control gene *GAPDH*, and ΔCT was calculated by subtracting the CT value of the *GAPDH* reference gene from that of each gene. Results were also expressed as fold changes (ΔΔCT) in the mRNA levels of a gene compared to the treated or untreated samples.

1.2.7 WESTERN BLOTTING

Protein expression levels of ß-catenin, the central component of canonical Wnt signaling, were determined by Western blotting. Equal amounts of cell lysate were resolved by SDS-PAGE, transferred to polyvinylidene difluoride membranes (Millipore), and detected with antibodies targeting either anti-human mouse ß-catenin mAbs at 1:500 dilution (R & D Systems, MN, USA), or anti-human mouse actin mAbs at 1:500 dilution (Millipore). Rabbit anti-mouse Horse Radish Peroxidase linked secondary antibodies (1:3000) were used to bind to the primary antibody. The blot was developed with enhanced chemiluminescence reagent (Pierce, IL) and images were captured using AlphaImager (CA, USA).

1.2.8 STATISTICAL ANALYSIS

Data represent mean and SE from experiments done in triplicate and were determined with a two-side Student's *t*-test, and the significant difference level was established at $P < 0.05$.

1.3 RESULTS

1.3.1 sFRP4 inhibits HNSCC spheroids at low dosage and increases the response with chemotherapeutics via the apoptotic pathway

To investigate the effect of sFRP4 on CSC enriched cells, we used two HNSCC cell lines. The effect of sFRP4 on CSC-enriched cells from the KB and Hep2 cell lines was tested with MTT and BrdU assays. It was observed that sFRP4 inhibited proliferation and, in combination with commonly used oral cancer chemotherapeutics such as cisplatin and doxorubicin, inhibition was more pronounced. The combination of cisplatin and sFRP4 effectively reduced the viability to 25% in both Hep2-spheroids and KB-spheroids (Fig 1a, b), and hence, cisplatin was used in the subsequent studies. The caspase-3/7 assay showed induction of apoptosis by the activation of caspase 3/7 enzyme and was maximal in the S+C treated cells (Fig 1c).

1.3.2 Self-renewal and CSC specific markers of Hep2-spheroids and KB-sheroids reduced by sFRP4

One of the strategies used for the enrichment of CSCs and for the analysis of self-renewal, a hallmark property of CSCs, is to study the formation of clonogenic spheres and secondary sphere formation. We have previously reported the formation of secondary spheres by the CSCs enriched from glioma cell lines (Warrier et al., 2012). We show that addition of sFRP4, and in combination with cisplatin, drastically disrupted the formation of secondary spheres (Fig 2a) and there was no clonogenic expansion. Expression of the CSC-specific marker CD44 was reduced markedly in sFRP4 treated and in sFRP-Cis treated Hep2-spheroids and KB-spheroids, as demonstrated by immunohistochemistry (Fig 2b). Flow cytometry showed a modest decrease in CD44 positive cells (Fig 2c).

1.3.3 Inhibition by sFRP4 is through the Wnt/ß-catenin signaling pathway

As sFRP4 is a known antagonist of the Wnt pathway, we used the Wnt agonist, Wnt3a, and the GSK-3ß inhibitor, lithium chloride, in our assay to see their effect on sFRP4-mediated inhibition. In sFRP4 treated cells, Wnt3a and LiCl increased proliferation of Hep2-spheroids and KB-spheroids and reversed the inhibition produced by sFRP4 (Fig 3a). Non-tumorigenic fibroblastic cells, MEFs, however, showed no effect upon various drug treatments. Further confirmation that the mode of action of sFRP4 is through the Wnt/ß-catenin axis was provided by Western blotting of sFRP4 and cisplatin treated Hep2-spheroids. It was seen that, after sFRP4 and cisplatin treatment, there was a marked reduction of ß-catenin protein, suggesting that sFRP4 abolishes accumulation of ß-catenin via the Wnt/ß-catenin signaling pathway (Fig 3b).

1.3.4 sFRP4 upregulates apoptotic genes and represses stemness genes

We studied expression of genes such as *Cyclin D1*, a regulator of cell cycle progression, *Bax*, a key promoter of apoptosis, and *p21*, a cyclin dependant kinase inhibitor, in Hep2-spheroids and KB-spheroids by q-PCR following treatment with sFRP4 and cisplatin. We found that treatment with sFRP4 in combination with cisplatin, activated *Bax* and *p21* gene expression but reduced the expression of *Cyclin D1*, proliferation markers *Ki67* and *MDM2*, and inhibitor of apoptosis, *xIAP* (Fig 4,b). As the CSC enriched cells had high levels of CSC-associated genes such as *CD44* and *ALDH*, we analyzed the expression of these genes under the various drug treatments. We found that all these genes were downregulated in sFRP4 and cisplatin treated spheroids derived from both Hep2 and KB cell lines (Fig 4a, b).

1.3.5 sFRP4 downregulated EMT promoting genes and drug resistance genes

Epithelial to mesenchymal transition (EMT), a typical trait of CSCs, is accompanied by a shift in cadherin expression from E-cadherin to N-cadherin. We found a reversal of EMT with the accumulation of E-cadherin and a reduction in N-cadherin levels in sFRP4, cisplatin, and combination treated Hep2-spheroids and KB-spheroids. In addition, we observed that the molecular markers implicated in EMT, such as *twist* and *snail*, were downregulated in spheroids treated with sFRP4 and cisplatin (Fig. 4a, b). The expression of the ABC genes *ABCG2* and *ABCC4*, which are involved in drug efflux and are normally upregulated in CSCs, were also analyzed and we observed that their expression was reduced in sFRP4-cisplatin treated Hep2-spheroids (Fig. 4 a, b).

1.4 DISCUSSION

Cancer stem cells have remained an elusive and ever changing population present within tumors. As their isolation from tumor tissue is a challenge, enrichment of CSCs from cell lines is an ideal alternative for the testing of novel drugs that can inhibit these highly refractory and chemoresistant populations. Due to their closeness to normal stem cells, CSCs rely on pathways that govern development, self-renewal, and cell fate. These pathways are largely regulated by three signaling programs; namely, the Wnt (Haegebarth and Clevers, 2009, Wang et al., 2010), Notch (Fan et al., 2010, Wang et al., 2010), and Hedgehog (HH) pathways (Dierks et al., 2008, Hofmann et al., 2009). Aberrant Wnt/ß-catenin signaling has been implicated in numerous malignancies attributed to the constitutive hyper-activation of the pathway. A level of regulation in normal cells that keeps ß-catenin levels in check is the presence of antagonists of the Wnt pathway, which either bind Wnt ligands or Wnt receptors, or both (Kawano and Kypta, 2003).

The sFRPs are structurally related to the frizzled (Fz) receptors and are antagonists of Wnt signaling functioning by interacting with both Wnts and Fz receptors (You et al., 2004, Lee et al.,2004). It has been shown that sFRP4 is involved in the regulation of apoptosis, proliferation, tissue formation, and tumor growth (Guo et al., 1998, Lacher et al., 2003, Feng et al., 2006, Hewitt et al., 2006). In this study, we show that similar to our early report on glioma CSCs (Warrier, 2014), sFRP4 was effective in improving the chemoresponse of CSC-enriched spheroids from HNSCC cell lines to the commonly used chemotherapeutic cisplatin.

As the sFRP4 mediated inhibition operates through the Wnt/ß-catenin signaling pathway, we could demonstrate that the inhibition by sFRP4 was rescued by the addition of a Wnt ligand such as Wnt3a. Inhibition by sFRP4 is relayed downstream by the activation of GSK3ß kinase and by the phosphorylation of ß-catenin. We could show that LiCl (an inhibitor of GSK3ß kinase), could also remove the inhibition brought about by sFRP4. Activation and accumulation of ß-catenin in the cytoplasm will result in the nuclear translocation of ß-catenin. In the nucleus, ß-catenin binds to the transcription factors of the T-cell factor- lymphocyte enhancing factor family, upregulating genes associated with proliferation and migration such as *cycD1*, *c-myc*, and *cox2*. Our observations of downregulation of the proliferation associated gene, *cycD1*, together with an

increase in the Wnt-regulated apoptotic genes, *Bax* and *p21*, indicate the initiation of the apoptotic process by suppression of Wnt signaling.

Wnt activity determines stemness and self-renewal of CSCs from colon cancers (Kaler et al., 2009) and breast cancers (Lamb et al.,2013), and WNT pathway activation is significantly higher in breast CSCs, whereas normal stem-like cells have lower levels of WNT signaling (Klopocki et al., 2004). Our data clearly indicate that sFRP4 is able to reduce the sphere forming and self-renewal ability of the CSC enriched HNSCCs. Furthermore, HNSCC-specific CSC markers such as CD44 and ALDH are downregulated by sFRP4 treatment. Pancreatic CSCs, identified by $CD44^{+}CD24^{-}$ and ALDH expression, have been shown to be sensitized to TNF-related apoptosis-inducing ligand (TRAIL)-induced apoptosis by sulforaphane, which acts by dysregulating anti-apoptotic NF-□B signaling (Kallifatidis et al., 2009). A similar mechanism may govern the dramatic reduction in self-renewal, clonogenicity, and CSC marker expression caused by sFRP4 treatment in conjunction with chemotherapeutic drugs in HNSCC spheroids.

EMT is a reversible dedifferentiation process converting epithelial cancer cells to dedifferentiated cells with mesenchymal properties, and is characterized by the loss of epithelial features and the acquisition of mesenchymal traits (Eccles and Welch, 2007, Thiery et al., 2009). The EMT transcription factors ZEB1/2 have been shown to be overexpressed in CSC-like cells in HNSCC and are linked to decreased survival rates, increased sphere formation, $CD44^{+}$ cells, tumor growth, and metastasis (Thiery, 2002). Loss of E-cadherin is the fundamental event in EMT. E-cadherin is a tumor invasion suppressor and is downregulated in most carcinomas, while N-cadherin, an invasion promoter, is frequently upregulated in the EMT switch. Upon EMT conversion, epithelial cells lose cell-cell adhesion and cell polarity, and have decreased expression of E-cadherin, and an increase in mesenchymal cell markers vimentin, fibronectin, N-cadherin, α smooth muscle actin (α-SMA), as well as increased activity of matrix metalloproteinases (MMPs) such as MMP-2, MMP-3, and MMP-9, which are associated with an invasive phenotype (Chu et al., 2013). The reversible downregulation of E-cadherin (encoded by *CDH1*) involves either hypermethylation of the *CDH1* promoter or repression by EMT-inducing transcription factors (Thiery and Sleeman, 2006, Thiery et al., 2009). The loss of E-cadherin releases β-catenin into the cytosol and elicits activation of the canonical Wnt signaling pathway

(Chu et al., 2013). An increased expression of E-cadherin over N-cadherin, which we observed following sFRP4 treatment, indicates a reversal of EMT. This phenomenon is further corroborated in our study by the downregulation of *Twist* and *Snail*, which are transcriptional repressors of E-cadherin (Chu et al., 2013).

ABC drug transporters are overexpressed in both normal stem cells and many CSCs as drug efflux pumps (Polyak and Weinberg 2009, Lou and Dean, 2007). Of the ABC transporters examined, we found a decrease in the expression of *ABCG2* and *ABCC4* in sFRP4 and drug treated spheroids. ABCG2 is an important member of ABC transporter family, and is one of the potential markers of CSCs that facilitate the mechanism of multidrug resistance (MDR), and is an important determinant of the CSC side population phenotype (Haraguchi et al., 2006, Lim et al., 2011).

The CSC assay technique used in this study, which utilizes CSC enrichment in stringent low attachment, serum-free culture, is an effective platform for multiple drug screening. We have shown that sFRP4, an endogenously expressed Wnt antagonist, is able to inhibit CSC growth and render these cells more responsive to chemotherapeutics. sFRP4 has no inhibition in normal non-cancerous cells because these cells have a low level of Wnt signaling. Hopefully, this study could provide an impetus for the emergence of sFRP4 as a viable inhibitor of CSCs in many tumors.

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DISCLOSURE OF INTEREST

Authors declare no potential conflicts of interest.

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FIGURE LEGENDS

Figure 1: Inhibition of HNSCC CSCs by sFRP4 and Cisplatin

a) MTT proliferation assay of Hep2 and KB spheroids showed an inhibition with sFRP4- 250pg/mL (S), doxorubicin- 50ug/mL (D), cisplatin- 10mM (C) , sFRP4 + doxorubicin (S+D), and sFRP4 + cisplatin (S+C).

b) Percentage of live cells was determined by a BrdU assay, which demonstrated that S+C treated cells had maximal cell death.

c) The caspase-3 assay showed that apoptotic cell death had occurred and was the highest in S+C treated cells.

Results are the mean ± SD of three independent experiments performed in triplicates (* p value <0.05, ** p value <0.01, n=3).

Figure 2: Analysis of CSC properties- sphere forming assay, immunohistochemistry, and CD markers analyses

a) Self-renewal by secondary sphere forming assay shows that sFRP4 treatment (S) disrupted sphere formation, and combination treatment (S+C) showed a marked reduction. (U) is the untreated control.

b) Immunohistochemistry of CSC specific marker CD44 of Hep2 and KB spheroids shows a similar pattern of reduction of CD44 expression. Nuclei have been stained with DAPI.

c) Flow cytometry of KB CSCs shows a decrease in CD44 expression.

Figure 3: Analysis of inhibition of sFRP4 effect by Wnt/β-catenin pathway regulators

a) Wnt agonist, Wnt3a, and GSK3-ß inhibitor lithium chloride (LiCl), increased proliferation of sFRP4 treated Hep2 and KB cells whereas all the drug treatment had no significant effect on non-tumorigenic mouse embryonic fibroblasts (MEF).

Results are the mean ± SD of two independent experiments performed in triplicates (* p value <0.05, ** p value <0.01, n=3).

b) Western blot analysis of ß-catenin in Hep2 and KB spheroids shows that ß-catenin expression is reduced in S and C treated cells, and almost abolished in S+C treated cells

Figure 4: Expression study of apoptotic genes, stemness genes, EMT and drug-resistant markers by semi-quantitative and quantitative RT-PCR

CyclinD1, *Xiap*, *MDM2* and *Ki67* levels, as represented by semi qPCR (a) quantified by qPCR (b), were reduced in S, C, and S+C treated Hep2 and KB spheroids, and the expression of apoptosis-inducing genes *Bax* and *p21* increased upon drug treatment. CSC-associated genes *CD44* and *ALDH* decreased, but *CD24*, a negative CSC marker, was shown to increase in both Hep2 and KB-CSCs (a, b1, b2). EMT-specific markers *Twist* and *Snail* decreased. Mesenchymal to epithelial switch was demonstrated by the decrease in N-cadherin and increase in E-cadherin (a, b1, b2). Decreased levels of drug resistance markers, *ABCG2* and *ABCC4* (a, b1, b2) were seen in the drug treated spheroids. A similar trend was observed in both Hep2 and KB-spheroids. (U) represents the untreated control. Results are the mean ± SD of two independent experiments performed in triplicates (* p value <0.05, ** p value <0.01, n=3).

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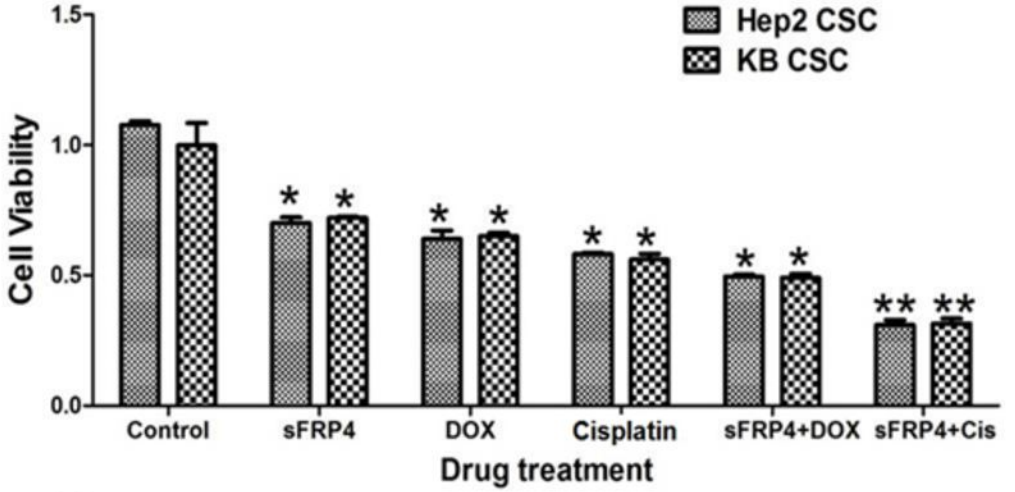
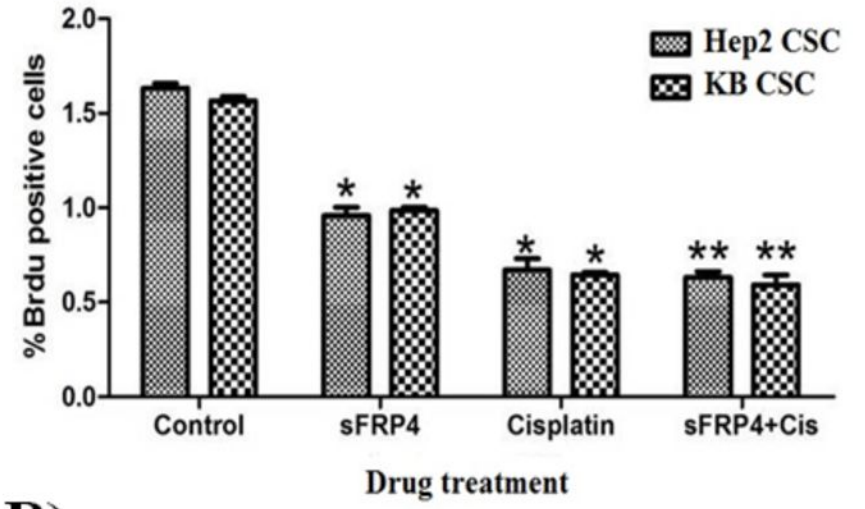
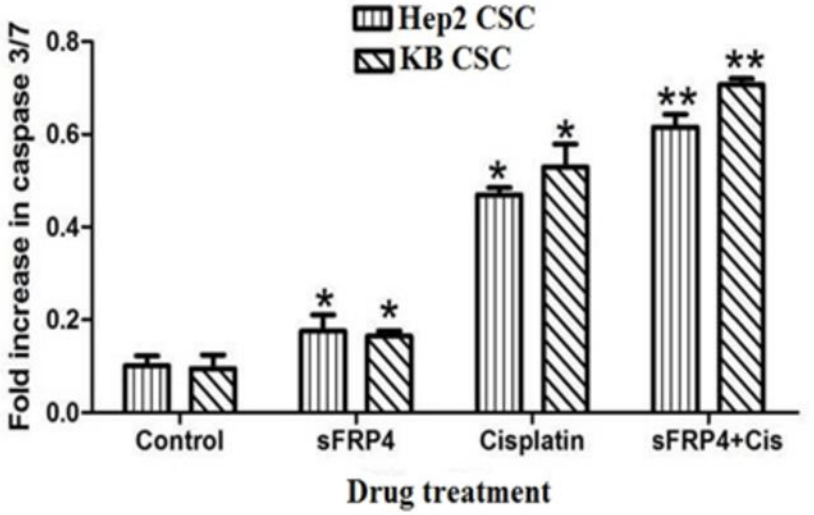

Hep2 CSC
KB CSC
Cell Viability
1.5
1.0
0.5
0.0
Control
sFRP4
DOX
Cisplatin
sFRP4+DOX
sFRP4+Cis
Drug treatment

A)

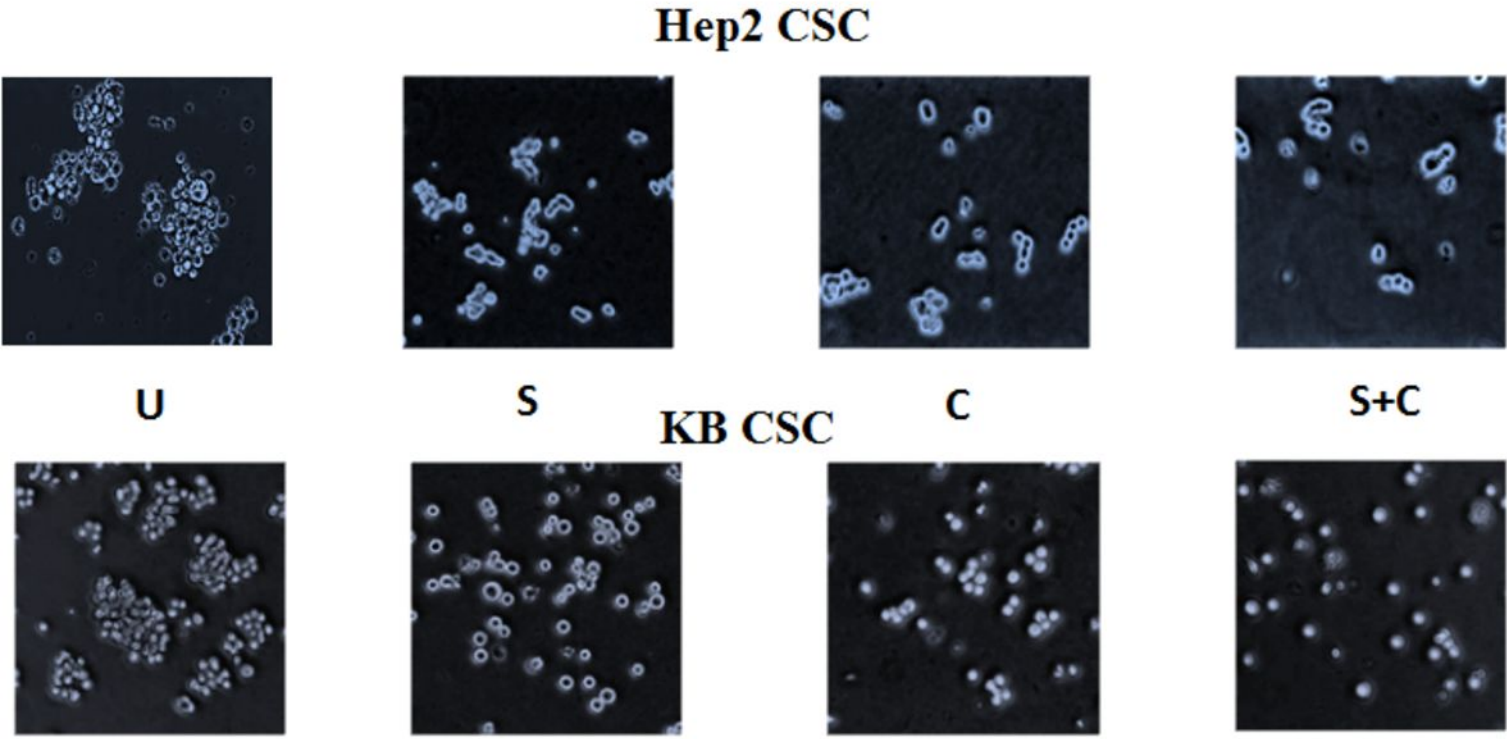

Hep2 CSC
KB CSC
% Brdu positive cells
2.0
1.5
1.0
0.5
0.0
Control
sFRP4
Cisplatin
sFRP4+Cis
Drug treatment

B)


Hep2 CSC
KB CSC
Fold increase in caspase 3/7
0.8
0.6
0.4
0.2
0.0
Control
sFRP4
Cisplatin
sFRP4+Cis
Drug treatment

C)

FIGURE 1



A)

FIGURE 2

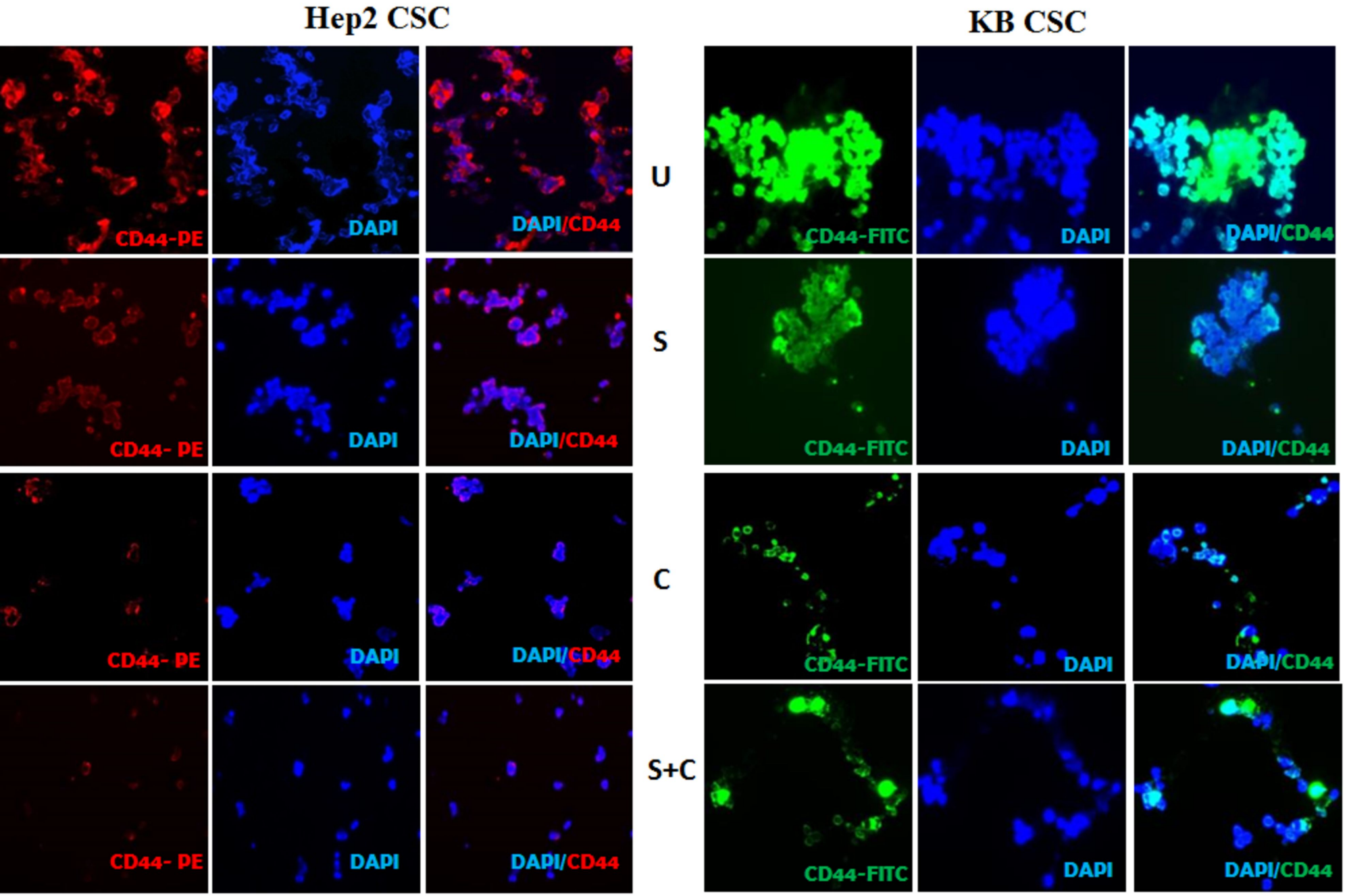


FIGURE 2 B)

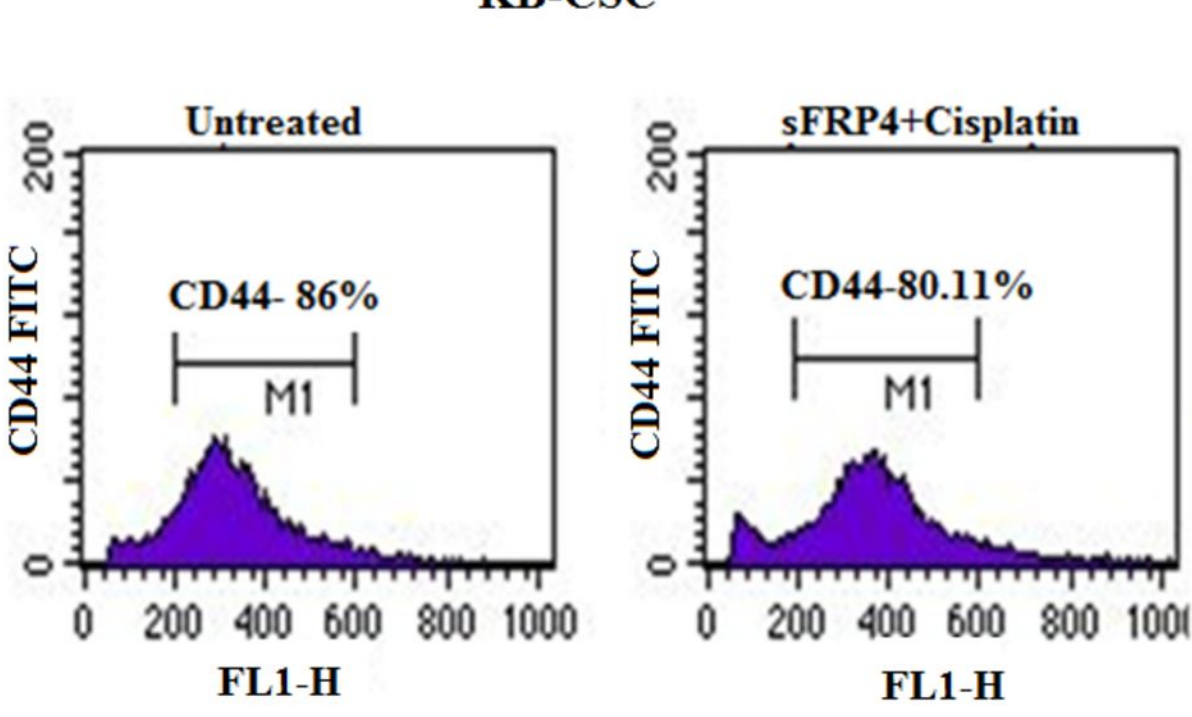
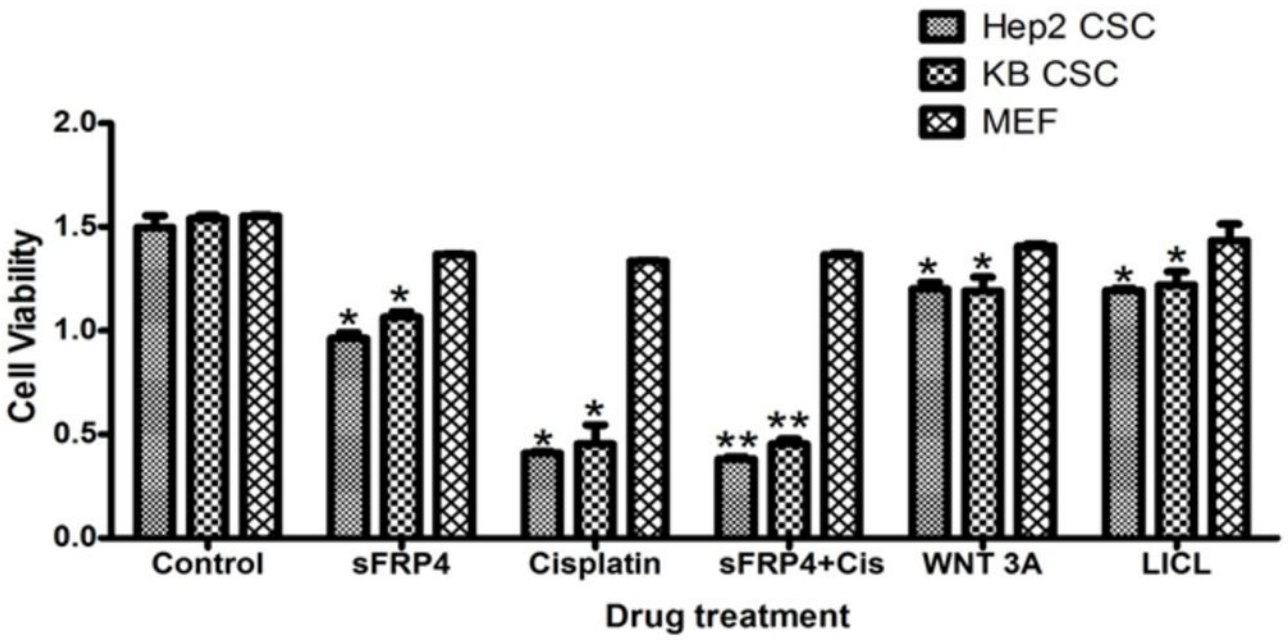
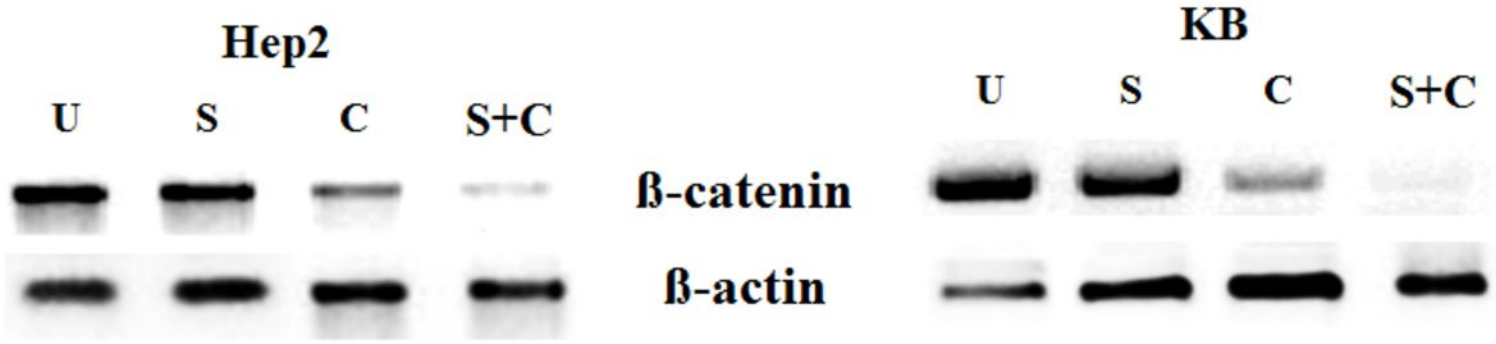

KB-CSC
Untreated
CD44 FITC
200
0
CD44- 86%
M1
0
200
400
600
800
1000
FL1-H
sFRP4+Cisplatin
CD44 FITC
200
0
CD44-80.11%
M1
0
200
400
600
800
FL1-H

FIGURE 2 C)

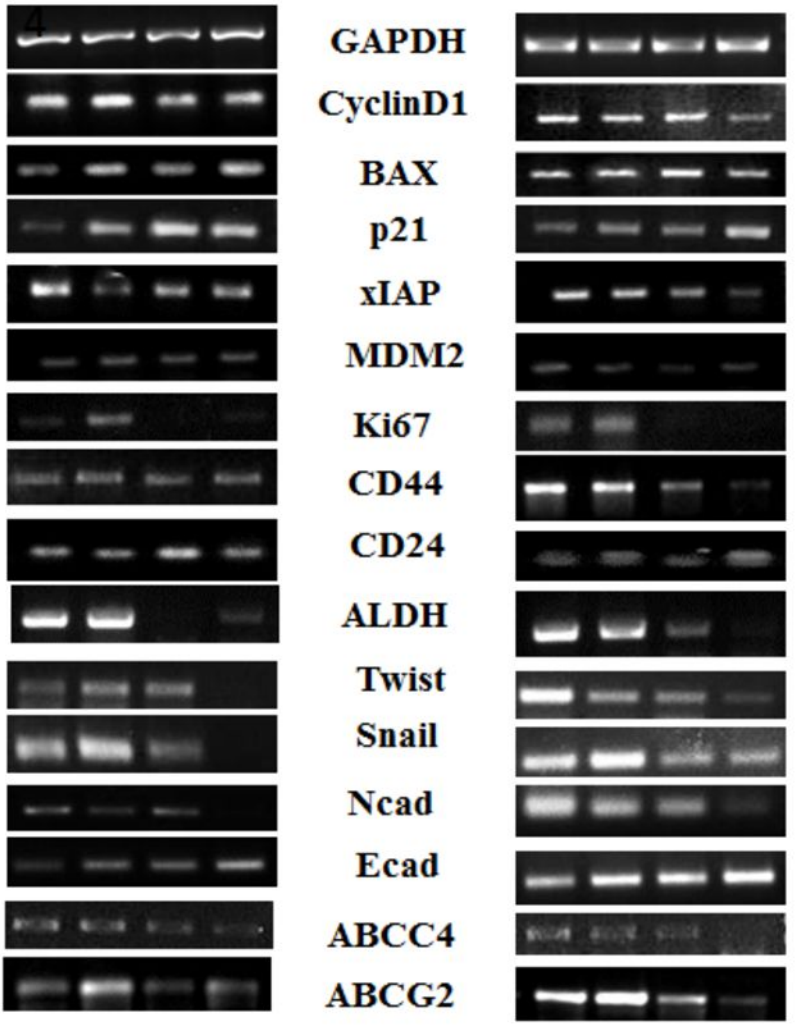
A)


Hep2 CSC
KB CSC
MEF
Cell Viability
2.0
1.5
1.0
0.5
0.0
Control
sFRP4
Cisplatin
sFRP4+Cis
WNT 3A
LICL
Drug treatment


Hep2
U
S
C
S+C
KB
U
S
C
S+C
ß-catenin
ß-actin

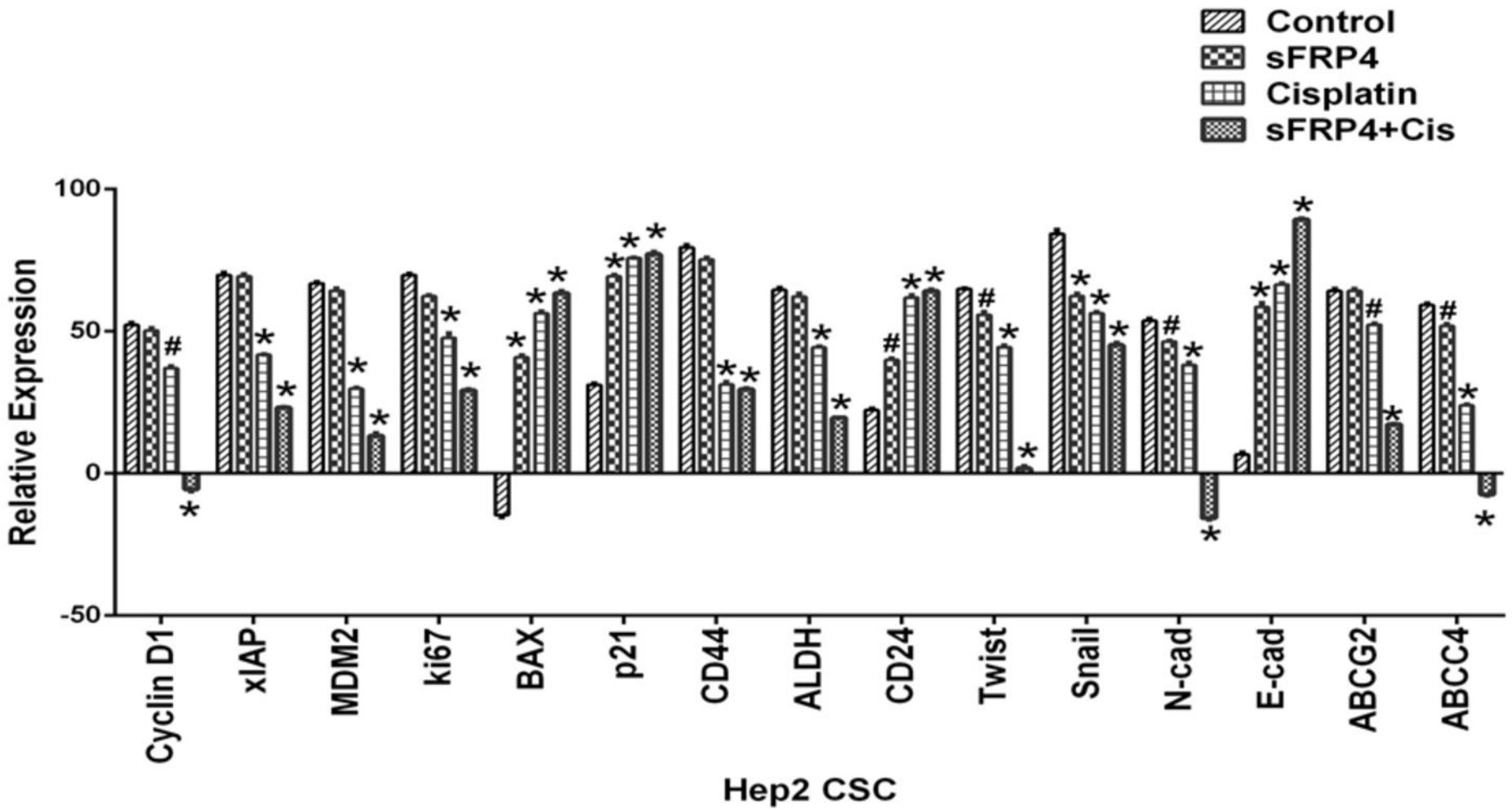
B)

FIGURE 3


Hep2 CSC
KB CSC
U S C S+C
U S C S+C
GAPDH
CyclinD1
BAX
p21
xIAP
MDM2
Ki67
CD44
CD24
ALDH
Twist
Snail
Ncad
Ecad
ABCC4
ABCG2

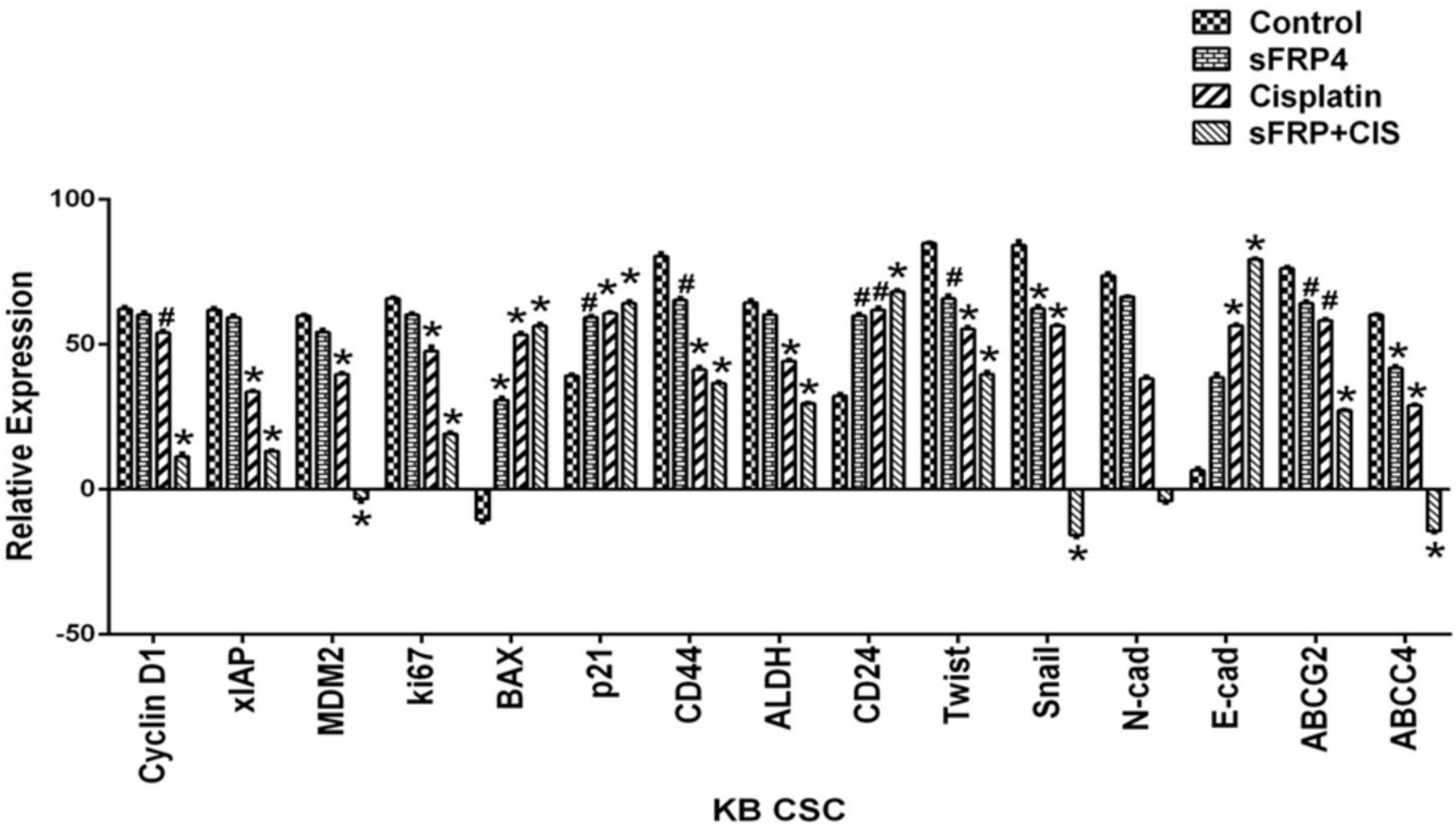
A)

FIGURE 4


Control
sFRP4
Cisplatin
sFRP4+Cis
Relative Expression
100
50
0
-50
Cyclin D1
xIAP
MDM2
ki67
BAX
p21
CD44
ALDH
CD24
Twist
Snail
N-cad
E-cad
ABCG2
ABCC4
Hep2 CSC

B1)

FIGURE 4


Control
sFRP4
Cisplatin
sFRP+CIS
Relative Expression
100
50
0
-50
Cyclin D1
xIAP
MDM2
ki67
BAX
p21
CD44
ALDH
CD24
Twist
Snail
N-cad
E-cad
ABCG2
ABCC4
KB CSC

B2)

FIGURE 4

Table 1- : Primer sequences for apoptosis and stemness related genes

Genes	Primers	Base pair	Annealing temperature ºC
GAPDH	F- 5' CAGAACATCATCCCTGCATCCACT 3' R- 5' GTTGCTGTTGAAGTCACAGGAGAC 3'	181	61
Cyclin D1	F- 5' AACTACCTGGACCGCTTCCT 3' R- 5' CCACTTGAGCTTGTTCACCA 3'	187	61
xIAP	F- 5'GGGGTTCAGTTTCAAGGACA3' R- 5' CGCCTTAGCTGCTCTTCAGT3'	183	56
Mdm-2	F- 5' TTACCCAGGCTGGAGTGCAG 3' R- 5' GAGAATGGTGCGAACCCG 3'	92	60
Ki67	F- 5 CTGCTTTGGGGACTTGACG 3' R- 5 GTCGACCCCGCTCCTTTT 3'	201	62
Bax	F- 5' GCTGGACATTGGACTTCCTC 3' R- 5' TCAGCCCATCTTCTTCCAGA 3'	247	61
p21	F- 5'GAGGCCGGGATGAGTTGGGAGGAG 3' R- 5' CAGCCGGCGTTTGGAGTGGTAGAA 3'	220	63
CD44	F- 5' CATCTACCCCAGCAACCCTA 3' R- 5'GGTTGTGTTTGCTCCACCTT 3'	271	56
ALDH	F- 5'AAAGTCAAAGGCTTCCTGCCC 3' R- 5'CTCCTGGAACACAGGTGACT 3'	191	60
CD24	F- 5' AACTAATGCCACCACCAAGG 3' R- 5' CCTGTTTTTCCTTGCCACAT 3'	188	55

Table 2- : Primer sequences for EMT and drug resistance associated genes

Genes	Primers	Base pair	Annealing temperature ºC
Twist	F- 5' CAGCGCACCCAGTCGCTGAA 3' R- 5' CGCCCCACGCCCTGTTTCTT 3'	438	53
Snail	F- 5′ GAGGCGGTGGCAGACTAG 3′ R- 5′ GACACATCGGTCAGACCAG 3′	159	60
N-cadherin	F- 5′CTCCTATGAGTGGAACAGGAACG 3′ R- 5′TTGGATCAATGTCATAATCAAGTGCTGTA 3′	121	63
E-cadherin	F- 5′ATTCTGATTCTGCTGCTCTTG 3′ R- 5′ AGTAGTCATAGTCCTGGTCTT 3′	400	60
ABCG2	F- 5' CACAGGTGGAGGCAAATCTT 3' R- 5'CCGAAGAGCTGCTGAGAACT 3'	199	60
ABCC4	F- 5' CCATCTGTGCCATGTTTGTC 3' R- 5' AGGGCTGAGATGAGGGAACT 3'	403	60