The Role of Secreted Frizzled Related Protein 4 (sFRP-4) in Regulating Oestradiol-Induced Growth of the MCF-7 Breast Cancer Cell Line

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Abstract: The Wnt signalling pathway is involved in regulating cellular proliferation and differentiation, and aberrant activation has been described in several cancers including breast. Oestradiol up regulates Wnt pathway gene expression, and thereby activates the Wnt signalling pathway. We used the oestrogen-responsive breast cancer cell line MCF-7 to examine the effects of secreted frizzled related protein 4 (sFRP-4) on oestradiol-induced growth, including gene expression of the Wnt signalling pathway genes Frizzled Receptor, Wnt-10b, and β-catenin. We demonstrate here that sFRP-4 inhibits oestradiol-induced cell growth in the MCF-7 cell line and also down regulates oestradiol-induced expression of selected Wnt signalling genes including β-catenin. We propose that sFRP-4 is a potent inhibitor of the Wnt signalling pathway and may negatively regulate oestradiol-mediated proliferation in human breast cancer cells.

Keywords: Breast cancer, sFRP4, Wnt signalling, oestradiol, β-catenin, cellular proliferation, growth inhibition.

INTRODUCTION

Reproductive tissues undergo cycles of hormone driven growth followed by regression involving apoptosis. A novel clone expressed in the rat corpus luteum undergoing regression was isolated by our laboratory [1, 2] and this gene, termed secreted Frizzled Related Protein-4 (sFRP-4), was shown by Northern blot analysis and *in situ* hybridization to be highly expressed in regressing ovarian, mammary gland, and ventral prostate tissues after hormone withdrawal [2]. sFRP-4 expression is undetectable in the resting mammary gland of the rat until involution, when there is a greater than ten-fold increase in sFRP-4 expression [2].

sFRP-4 is related to the frizzled (FZ) family, which functions as a receptor for the ubiquitous family of secreted Wnt signalling molecules. Based on amino acid sequence analysis, the FRP cDNA is believed to encode for the frizzled-like, cysteine-rich domain but not the seven-pass transmembrane domain seen in other FZ family members [3]. Thus, it has been proposed the FRPs are secreted proteins that compete for Wnt ligand binding, as well as forming a dominant negative interaction with the FZ receptor to modulate the Wnt-frizzled signal transduction pathway [4].

Northern blot analysis of a variety of human tissue RNA samples has shown that there are high levels of expression of sFRP-4 in ovary, prostate, testis, spleen, and thymus, but not in the colon, small intestine, and peripheral blood leukocytes. To date, only one published study has examined sFRP-4 expression in human breast tissue and breast tumours using *in situ* hybridization [5]. They identified an up regulation of sFRP-4 mRNA in *in situ* and invasive ductal breast carcinomas compared with the normal breast parenchyma [5]. The induction of sFRP-4 expression in the *in situ* carcinoma, the earliest form of breast tumour, suggests its expression may be an early neoplastic event [5]. Oestrogen has been implicated as a potential regulating factor of sFRP-4 due to the predominant expression of sFRP-4 during the oestrogenic phase of the endometrial cycle [5].

SFRP-4 expression has been demonstrated in prostate carcinomas compared with normal prostate

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tissue [6]. Membranous expression of sFRP-4 was associated with good patient prognosis, highlighting its potential as a prognostic marker and suggesting that sFRP-4 may be involved in counteracting unregulated growth in hormone-responsive cancers [6]. More recently, sFRP4 expression was associated with a better prognosis in ovarian cancer patients and acts through inhibition of the Wnt signalling pathway [7, 8]. It was found that over expressing sFRP4 in breast cancer cell lines that were resistant to cisplatin rendered these cells sensitive to treatment [7]. Furthermore, there was a positive association between decreased sFRP4 expression and increased severity of tumour seen in patient tissue microarrays [7]. In the present study we examined the role of sFRP-4 on the growth of the breast cancer cell line MCF-7, specifically examining whether it was able to modulate oestradiol-induced cell growth.

MATERIALS AND METHODS

Cell Culture

The human breast cancer cell line, MCF-7 (donated by Dr Jacky Bentel), was grown in RPMI 1640 (Gibco) supplemented with 10% foetal bovine serum (FBS) (JRH Biosciences), 2mM L-glutamine (L-glut) (Gibco), 2mM penicillin (Gibco), and 2mM streptomycin (Gibco). Cells were cultured at 37°C with 5% CO_2. Trypsinised MCF-7 cells were seeded onto 6-well tissue culture plates (Sarstedt) at a final concentration of 5 x 10^4 cells per well for all experiments. Cells were incubated overnight to allow attachment to the well surface. Seeding day was allocated as day one.

Oestradiol Treatment

Phenol red-free (PRF) RPMI medium (Gibco) supplemented with 5% charcoal-stripped serum (Trace), 2mM L-glutamine (L-glut), 2mM of penicillin and streptomycin were added to the cells on day two to starve the cells of oestrogens. 100nM of 17β-oestradiol (E_2) (Sigma) were added to the cells on day four together with fresh PRF charcoal-stripped growth medium. Cells were incubated with E_2 for 72 hours.

sFRP-4 Conditioned Media Experiments

NIH:3T3 cells (derived from embryonic mouse fibroblasts and obtained from ATCC, Virginia, USA) were virally transfected with the sFRP-4 gene as previously described (Lacher *et al*. 2003). 3T3 cells were maintained in RPMI 1640 supplemented with 10% FBS, 2mM L-glut, 2mM penicillin, and 2mM streptomycin. The sFRP-4 conditioned media were aspirated and used in the sFRP4 treatment experiments. SFRP-4 protein concentration in the conditioned media was determined using a standard Bradford assay. Cell media were aspirated from untransfected 3T3 cells to use as a control for sFRP-4 treatment.

Determining sFRP-4 Dose

Protein isolation was performed on the sFRP-4 transfected (NIH:3T3+sFRP-4) and untransfected 3T3 cell media to determine the total protein concentration of the media and, thus, approximate a dose of total protein for conditioned media treatment.

10^6 NIH:3T3 and NIH:3T3+sFRP-4 cells were seeded in separate T-25 culture flasks in RPMI, 10%, FBS, and 2mM penicillin and streptomycin antibiotics and left to adapt to the culture flask overnight. To ensure the conditioned media for treatment were not dominated by the proteins present in FBS, media were replaced with serum-free media and the cells cultured for 72 hours. Protein was extracted from the media following three days of culture. Medium was passed through a 0.45µm filter and two 3ml aliquots of each sample were transferred to 15ml falcon tubes. The unused media were stored at 4°C for future treatments. 12ml of ice-cold acetone were added to each of media samples, the samples vortexed and then incubated at -20°C for 60 minutes. Samples were then centrifuged at maximum speed at 4°C for 10 minutes to pellet the protein precipitate. The supernatant was removed and discarded and the acetone evaporated from the samples by leaving the tubes open at room temperature for 30 minutes. The protein pellet was then re-suspended in 50µl of 0.01X PBS, the two samples pooled together, and an aliquot set aside for the Bradford protein assay to determine total protein concentration.

A 350µl dose of conditioned media from both transfected and untransfected cells was used for MCF-7 treatment. The protein concentration of media samples was determined by Bradford protein assay, and this dose represents approximately 1.3mg of total protein for untransfected cell media and 1.6mg for 3T3+sFRP-4 cell medium. The 300µg difference between these two samples potentially represents the amount of sFRP-4 present in the 3T3+sFRP-4 culture medium. The volume of conditioned media was kept constant for the MCF-7 treatments to control for the presence of depleted media in culture.

sFRP-4 Treatment

PRF RPMI medium supplemented with 5% charcoal stripped serum, 2mM L-glutamine, 2mM penicillin and streptomycin was added to the MCF-7 cells on day two. 350µl of sFRP-4 conditioned media were added to the treatment cells on day four together with 2mls of fresh PRF charcoal stripped growth medium. 350µl of 3T3 untransfected cell media were added to the control cells together with 2mls of fresh PRF charcoal stripped media, to account for the addition of depleted growth media on the cells. Cells were incubated with sFRP-4 for 72 hours.

Combined Oestradiol and sFRP-4 Treatment

A combined E_2 and sFRP-4 treatment was undertaken to investigate whether sFRP-4 can counteract oestradiol-induced proliferation of MCF-7 cells. These were performed in substantially the same way as described above except 350µl of sFRP-4, conditioned media were added to the cells on day four together with 2mls of fresh PRF charcoal stripped growth medium and 100nM E_2, and then incubated for 72 hours. Controls received 350µl of 3T3 untransfected cell media together with 2mls of fresh PRF charcoal stripped media and 100nM E_2.

Cell Proliferation Assay

Following the 72 hour treatments of E_2, sFRP-4 conditioned media, and combined E_2 and sFRP-4 conditioned media, cells were trypsinised and counted using a haemocytometer (Nebauer Improved, Crown Scientific). To ensure accurate counting, each sample (n=3 for each sample) was loaded into the haemocytometer twice, and in each load, three representative 25-square areas were counted, resulting in six counts per sample.

Real-Time RT-PCR

RNA was extracted from MCF-7 cells using TRI REAGENT according to the manufacturer's instructions (Molecular Research Centre). RNA concentration was determined from the absorbance at 260nm. 1µg of each RNA sample was DNase-treated using the DNA-*free*™ kit (Ambion) according to manufacturer's protocol to remove any genomic DNA contamination. RNA was reverse transcribed into cDNA, using M-MLV reverse transcriptase (Promega) according to a standard protocol. Point mutant #M3682 (Promega) was added to each sample. Clean-up for the reverse transcription reaction samples was completed using the Ultraclean PCR Clean-up DNA purification kit (MO BIO) in accordance with manufacturer's protocol.

The real-time PCR reaction mix consisted of 5µl of iQ™ SYBR® Green Supermix (BIORAD), 1µl each forward and reverse primers (5µM) (table 2.1), 2µl of dH_2O, and 1µl of cDNA to make a total reaction volume of 10µl. 1µl of water was added instead of cDNA in negative (no-template) controls. Samples were measured in duplicates. The PCR reaction was performed on a Rotor-Gene RG-3000 (Corbett Research). Cycling conditions varied for each gene and are detailed in Table **1**. A fluorescence measure was taken at the end of each cycle to form an amplification curve. The starting amount of cDNA template was extrapolated from Ct values and was the basis for calculating and comparing mRNA expression for each sample. Quantitation values were normalized to the housekeeping gene L-19, determined by the absorbance reading at 260nm. Amplified product obtained from the PCR reaction was run on a 2% agarose gel to determine product size.

Immunoblotting

Protein was extracted from MCF-7 cells by incubating with 100µl of RIPA buffer for 30 min on ice. The samples were centrifuged at maximum speed for 5 minutes at 4°C. The concentration of the protein supernatant was determined by BSA assay. 50µg of protein were separated by SDS gel electrophoresis on a 10% polyacrylamide gel, and then transferred onto a Hybond™-C nitrocellulose membrane (Amersham). Membranes were blocked in 0.5% (v/w) gelatin solution for 30 minutes at 37°C. To detect sFRP-4 protein expression, rabbit polyclonal anti-rat sFRP-4 (Upstate Biotechnology, USA) was applied to the membranes. The human and rat proteins share 90% homology (Pubmed library: rat: NP_445996, human: NP_003005). The sFRP-4 antibody was diluted 1:250 in TBST-0.05% and incubated with the membrane overnight at 4°C with shaking. Membranes were then incubated with HRP-conjugated goat anti-rabbit IgG (DAKO) diluted 1:5000 for 1 h at room temperature. All signals were detected using enhanced chemiluminescence system (SuperSignal® West Pico Chemiluminescent substrate PIERCE).

The activity of the Wnt signalling pathway was measured by quantifying the levels of dephosphorylated β-catenin. Mouse anti-active β-catenin antibody (Upstate™) was diluted 1:330 in TBST-0.05% and incubated with the membrane

Table 1: Gene Primers and Thermocycling Conditions for Genes Examined

Gene	Primer Sequence	Cycling conditions	Cycles	Product Size
sFRP-4	F 5' TCT GTA CCA AAG GGC AAA 3' R 5'ACC ACC GTT GTG ACC TCA TT 3'	94°C for 3mins 94°C for 30sec 60°C for 30sec 72°C for 30sec	45	110bp
Fz-4	F 5' GAC AAC TTT CAC ACC GCT CA 3' R 5' GGC AAA TCC AAA TTC CTT CA 3'	94°C for 3mins 94°C for 30sec 55°C for 30sec 72°C for 30sec	45	178 bp
Wnt-10b	F 5' TGG GCC GGG CCA TCT TCA TT 3' R 5'GGC TGC CAC AGC CAT CCA AC 3'	94°C for 3mins 94°C for 30sec 60°C for 30sec 72°C for 60sec	45	204 bp
β-catenin	F 5' GAT TTG ATG GAG TTG GAC 3' R 5' TGT TCT TGA GTG AAG GAC 3'	94°C for 3mins 94°C for 30sec 52°C for 30sec 72°C for 30sec	45	218 bp

overnight at 4°C with shaking. Membranes were then incubated with HRP-conjugated rabbit anti-mouse IgG (DAKO) diluted 1:5000 for 1 h at room temperature.

Membranes were stripped of antibody by incubating with stripping buffer for 30 minutes at room temperature, and then re-probed with the house-keeping protein β-actin. The membranes were blocked in skim milk powder for 30 min at 37°C followed by incubation with the β-actin antibody (Sigma) (1:5000) overnight at 4°C with shaking. Membranes were then incubated with secondary HRP-conjugated rabbit anti-mouse IgG (1:10000) for 1 h at room temperature.

Western blots were quantified using Scion Image software by analysing the pixel density between the control and treatment samples. Data were normalised by dividing quantitation values by the β-actin quantitation values to account for any error in loading or variation in protein concentration.

Statistical Analysis

Data are represented as mean values ±SEM. Differences between groups were analysed by an un-paired two-tailed *t*-test with equal variances, and were considered statistically significant when $p<0.05$.

RESULTS

We used a cell counting method to establish proliferation rates of MCF-7 cells grown in culture. When MCF-7 cells were treated with 17β-oestradiol for a 72 hour period, we observed a 25% increase in cell number compared to the ethanol control ($p<0.001$; Figure **1A**). When MCF-7 cells were incubated with sFRP-4 alone, there was a statistically significant but relatively modest 15% reduction in MCF-7 cell number by comparison with untreated cells ($p=0.003$; Figure **1B**). Finally, when sFRP-4 was added in combination with oestradiol, we observed that the increase in the number of cells we might have expected was reduced about one half when compared to oestradiol treatment alone ($p<0.001$; Figure **1C**). Taken together, these results suggest sFRP-4 antagonises MCF-7 cell proliferation both with and without oestradiol driving that growth.

To ensure that oestradiol was not affecting endogenous sFRP-4 levels, we examined both mRNA and protein expression of sFRP-4 in MCF-7 cells for all treatments. No statistical difference was observed between either endogenous sFRP-4 mRNA or protein levels in any of our experiments (Figure **2**).

Since oestradiol has been previously described as stimulating expression of Wnt pathway associated genes in MCF-7 cells, and we had observed that sFRP-4 could suppress oestradiol-induced cellular proliferation, we undertook to quantitate expression of these genes to determine if sFRP-4 was suppressing their expression. We selected Wnt-10b for RT-PCR analysis because this has been previously associated with oestradiol-induced Wnt pathway stimulation [9-11]. All treatments were for 72 hours and, in all PCR

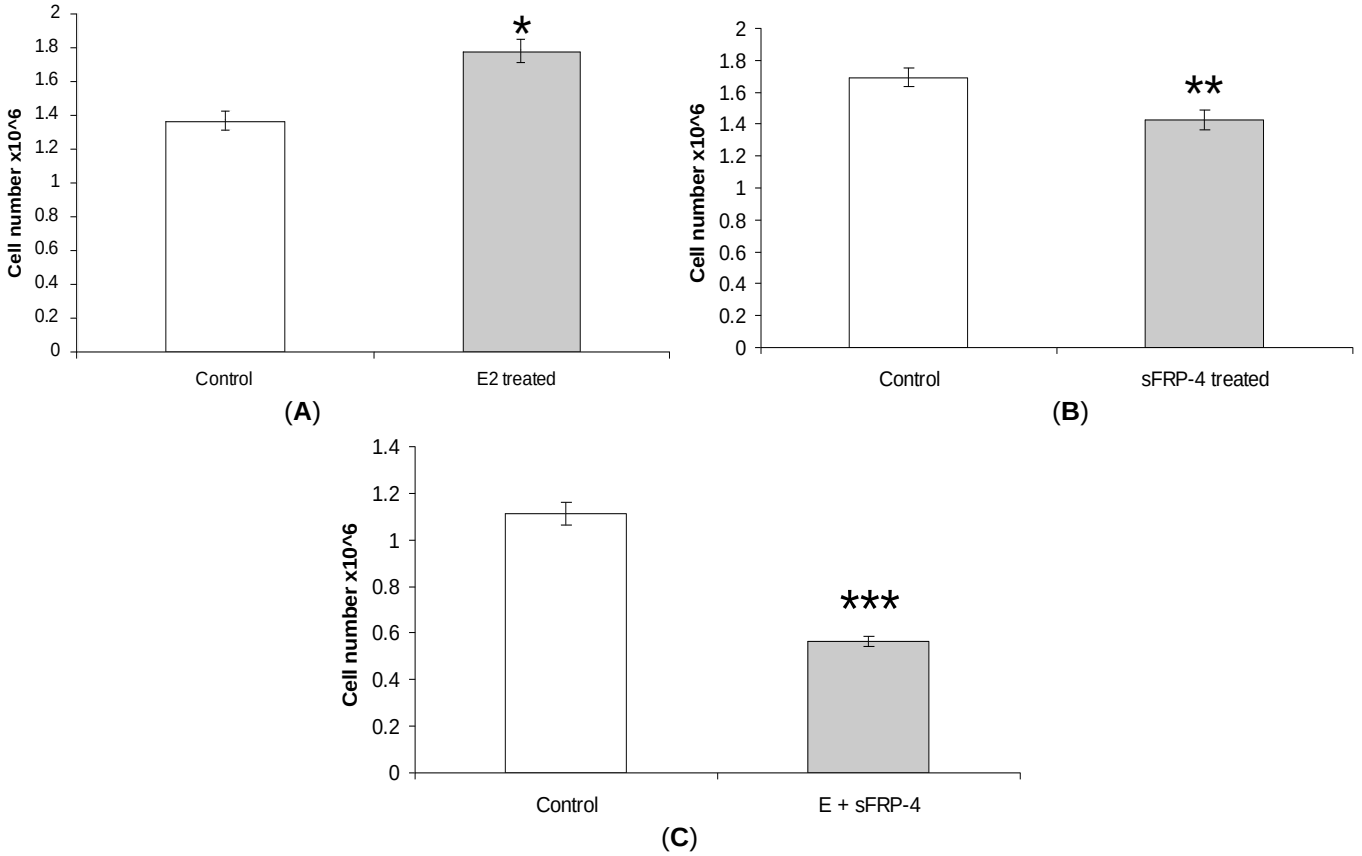


Figure 1: MCF-7 cell counts following 72hrs of treatment.

(**A**) Cells treated with E_2 for showed a 25% increase in cell number compared to controls (*p<0.001). (**B**) Exogenous sFRP-4 treatment resulted in a 15% reduction in MCF-7 cell number (**p=0.003). (**C**) Combined treatment of E_2 and sFRP-4 resulted in a 50% reduction in MCF-7 cell number (***p<0.001). Values indicate the average total number of cells as determined by haemocytometer counts ± SEM. A two-tailed t-test confirmed statistical significance between the two treatment groups (n=4 per treatment group).

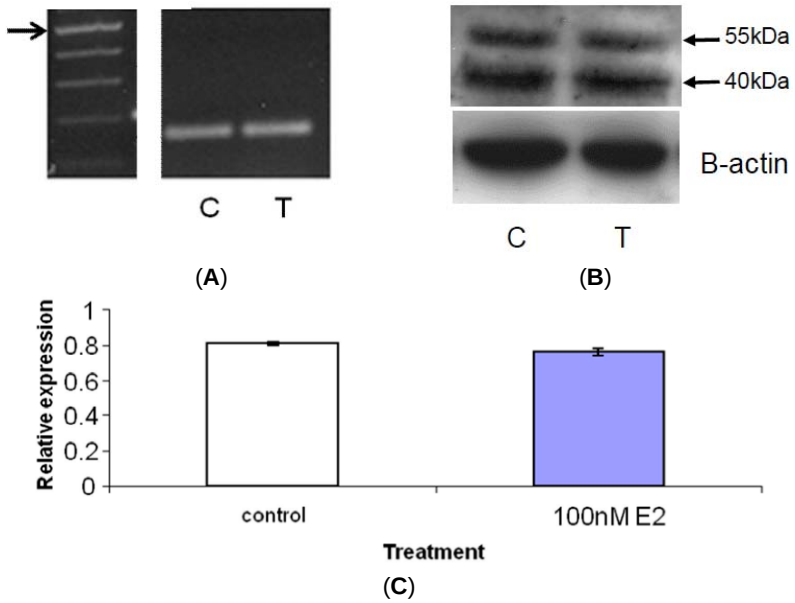
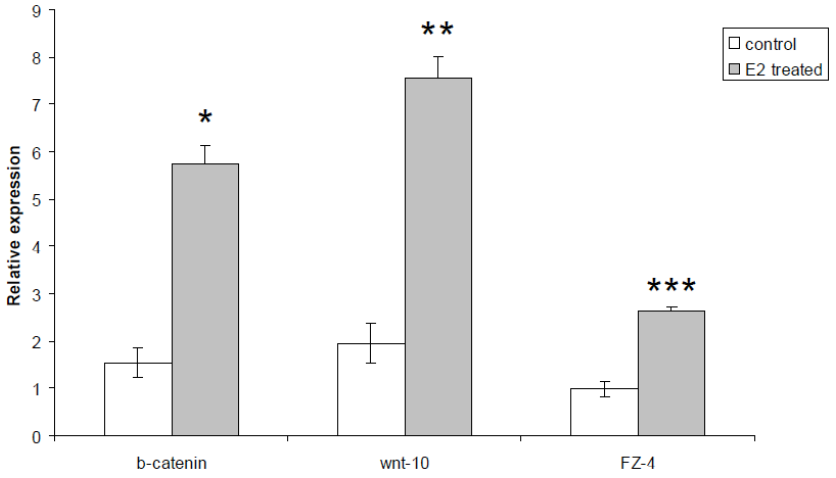
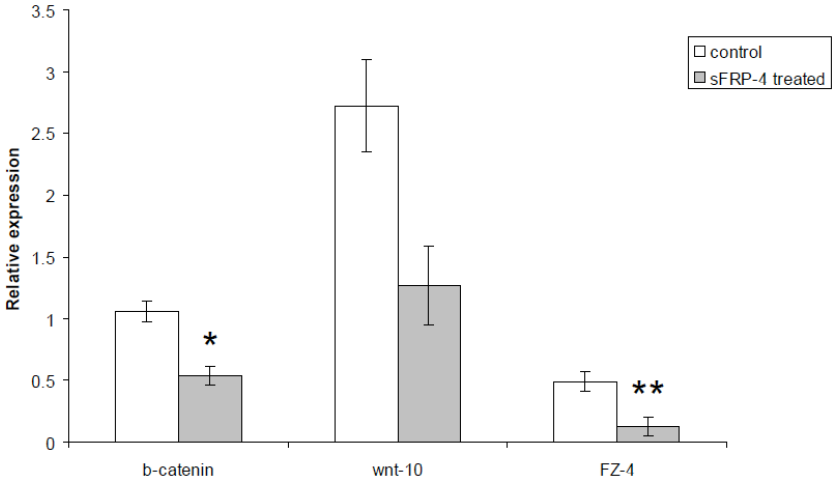


Figure 2: Effect of 72hrs of oestradiol treatment on endogenous sFRP4 mRNA and protein levels.

(**A**) Lane one is a 100 base pair ladder, arrow indicates 500kb, bands decrease in 100bp increments. Lane two represents control (C) and E_2 treatment (T) sFRP-4 PCR product 110bp in length (dissected from the gel). (**B**) Western blot demonstrating control (C) and E_2 treatment (T) bands of sFRP-4 protein. sFRP-4 antibody detected a 40kDa protein and 55kDa glycosylated sFRP-4 protein and β-actin (dissected from the gel). (**C**) Quantitation by densitometry, followed by normalisation to β-actin protein expression showing no change in sFRP4 protein levels (n=4).



(**A**)



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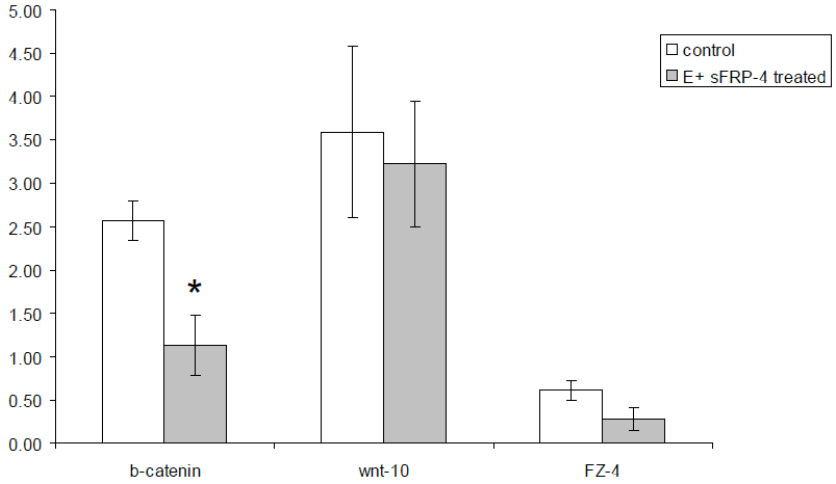


(**C**)

Figure 3: Quantitation graph of Wnt signalling genes following treatments.

Values represent the mean relative mRNA expression averaged from four samples ± SEM as determined by comparative quantitation analysis. Values were normalised to the housekeeping gene L-19. Asterisks denote significance as determined by a two-tailed t-test. (**A**) Quantitation graph of β-catenin, Wnt-10b, and FZ-4 mRNA following E_2 treatment for 72hrs. Treatment resulted in a significant increase in the mRNA of all three genes ($p<0.001$ for all genes). (**B**) Quantitation of Wnt signalling genes following exogenous sFRP-4 treatment for 72hrs. Treatment caused a significant reduction in both β-catenin and FZ-4 mRNA ($p=0.005$, $p=0.039$ respectively). (**C**) Quantitation of Wnt signalling genes following 72hrs of combined E_2 and sFRP-4 treatment. A significant reduction in β-catenin was apparent following combined treatment ($p=0.001$). The graphical data represent the mean values ±SEM of treatment groups (n=4).

reactions, a single peak was detected on the melt curve indicating the amplification of a single PCR product. Negative controls did not show product amplification (data not shown).

E_2 Treatment

Oestradiol treatment resulted in a highly significant four-fold increase in Wnt-10b mRNA, a 3 fold increase in β-catenin mRNA, and a 2.7 fold increase in FZ-4 mRNA levels by comparison with control ($p<0.001$, Figure **3A**).

A reduction in the levels of Wnt-10b mRNA showed a trend towards significance ($p=0.06$; Figure **3B**). SFRP-4 treatment resulted in a 50% reduction in β-catenin mRNA levels ($p=0.002$) and also induced a statistically significant 70% reduction in FZ-4 mRNA levels by comparison with control ($p=0.03$; Figure **3B**).

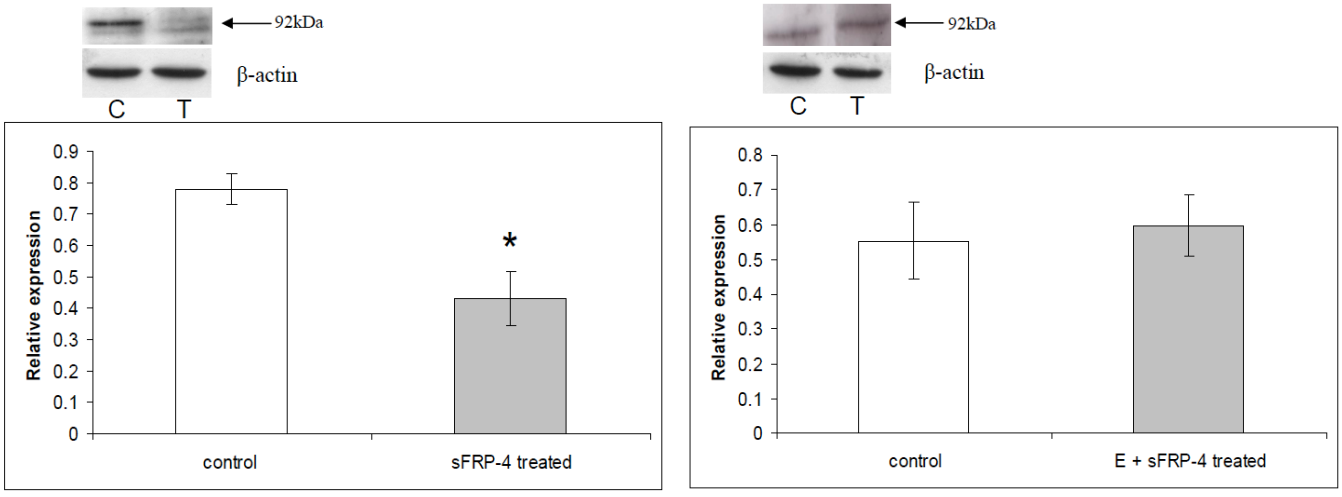
The combined treatment resulted in suppression of the oestradiol-induced expression of Wnt-10b and FZ-4, but this was not statistically significant for either Wnt-10b or FZ-4 ($p=0.8$, $p=0.1$ respectively; Figure **3C**). However, a significant reduction in β-catenin mRNA levels was detected in treated cells ($p=0.01$).

Whilst we had demonstrated a suppression of expression of both ligand and receptor of Wnt signalling pathway genes by sFRP-4 in MCF-7 cells, it was not clear if this actually resulted in a suppression of Wnt signalling itself. Therefore we investigated this by using an antibody that is specific to the active dephosphorylated form of β-catenin. A 92kDa protein band was detected in both control and treated protein samples, and was not detected in no-primary antibody controls (Figure **4**). Exogenous sFRP-4 treatment resulted in a highly significant halving of the levels of active β-catenin protein in MCF-7 cells ($p=0.01$; Figure **4A**), indicating a suppression of the Wnt signalling pathway. A slight, non-significant increase in active β-catenin protein levels followed combined E_2 and sFRP-4 oestradiol treatment ($p=0.77$; Figure **4B**).

DISCUSSION

Oestradiol is able to induce proliferation in the breast cancer cell line MCF-7, and there is evidence that this is mediated through the Estrogen Receptor (ER) acting as a transcription factor controlling the up regulation of genes involved in progression through the cell cycle, such as *c-myc* and *cyclin D1* [12]. Oestradiol also results in the up regulation of selected Wnt genes [13-19], although whether these are under the direct control of the ER has not been demonstrated since the promoter region of these genes does not have Estrogen Response Element sequences.

In prostate cancer cells, sFRP-4 was associated with a better prognosis and, in *in vitro* experiments,



(**A**) (**B**)

Figure 4: (**A**) Active β-catenin protein levels were measured following 72hrs of sFRP-4 treatment to determine its effect on Wnt signalling activity. Western blot demonstrating control (**C**) and treatment (T) band of 92kDa active β-catenin and β-actin (dissected from the gel). Quantitation by densitometry, followed by normalisation to β-actin protein expression, demonstrated a 55% reduction in the levels of active β-catenin protein compared to the control following sFRP-4 treatment (*$p=0.01$; two-tailed t-test). (**B**) Active β-catenin protein levels were determined following 72hrs of combined E_2 and sFRP-4 treatment. Western blot demonstrating control (**C**) and treatment (T) band of 92kDa active β-catenin and β-actin (dissected from the gel). Analysis revealed no significant difference between the levels of active β-catenin protein in control or combined treated cells ($p=0.77$). The graphical data represent the mean values ±SEM of treatment groups (n=4).

over expression of the sFRP-4 protein was able to down regulate the canonical Wnt signalling pathway [6]. Wnt-mediated signalling leads to an increase in the active form of cytosolic β-catenin, which in turn translocates to the nucleus where it is involved in co-receptor activity with T-cell factor/Lymphoid enhancer factor (TCF/LEF) transcription factors [18]. This pathway is also associated with enhancing the transcription of Wnt target genes such as *c-myc* and *cyclin D1* [19].

Previous studies have demonstrated that transcription of some Wnt genes is induced by oestradiol in MCF-7 cells [11, 13, 14, 16, 17]. However, none of these studies demonstrated whether this up regulation led to an increase in Wnt signalling activity or in cell proliferation. In the present study, oestradiol was able to stimulate proliferation in MCF-7 cells and also increase the expression of selected Wnt signalling pathway genes. We chose Wnt-10b as a candidate Wnt gene as it has been previously reported to be up regulated following oestradiol treatment [11], and has been implicated in human breast cancer [9]. We also examined the expression of FZ-4 since we have previously shown it to interact with sFRP4 in the rodent ovary [20]. RT-PCR analysis demonstrated a significant oestradiol-induced increase in the expression of Wnt-10b ($p<0.001$), consistent with previous data [11]. In addition, we observed an increase in the expression of mRNA for the Wnt receptor FZ-4 as well as an increase in the downstream Wnt signalling marker β-catenin ($p<0.001$ for both genes), suggesting that oestradiol was associated with activation of the Wnt signalling pathway in MCF-7 cells. However, we did not observe an increase in the active form of the β-catenin protein, in contradiction of the mRNA expression seen following oestradiol treatment (data not shown). These results seem to suggest that either oestradiol modulates the expression of active-β-catenin to steady-state levels, or that oestradiol induces MCF-7 proliferation by a mechanism independent of the Wnt signalling pathway. Alternatively, active-β-catenin levels may be increasing at a later time point, which may then act to increase cell proliferation but alternative oestrogen signalling pathways are able to induce proliferation earlier. Oestradiol is known to regulate the expression of genes involved in cell-cycle progression such as *c-myc* and *cyclin* [12]. It is likely that oestradiol induced MCF-7 cell proliferation is brought about by up-regulating the expression of these cell-cycle genes and that the Wnt signalling pathway is either activated at a later time-point, or is not regulated by oestradiol and plays no part in oestrogen-induced cellular proliferation in MCF-7 cells. In support of the antagonistic role of sFRP-4 to oestrogen-driven cell proliferation, it has recently been shown that sFRP-4 is able to down regulate the expression of *c-myc* and *cyclin D1* [21].

As predicted, exogenous sFRP-4 treatment significantly reduced MCF-7 cell proliferation, both under normal growth conditions and when stimulated by oestradiol. When treated with sFRP-4 alone, we observed a decrease in cell proliferation and this was associated with a decrease in both the levels of mRNA and the active form of the β-catenin protein. This observation is consistent with the proposed model of sFRP-4 antagonism of the Wnt signalling pathway, whereby its binding to Wnt prevents an association with the FZ receptor or formation of a non-functional complex with the FZ receptor [4, 6, 22]. This implies that, in untreated MCF-7 cells, normal cell growth requires an active Wnt signalling pathway or that this pathway is able to interfere with normal growth. Figure **5A** represents a proposed model of the interaction between sFRP-4 and oestradiol on the MCF-7 breast cancer cell line.

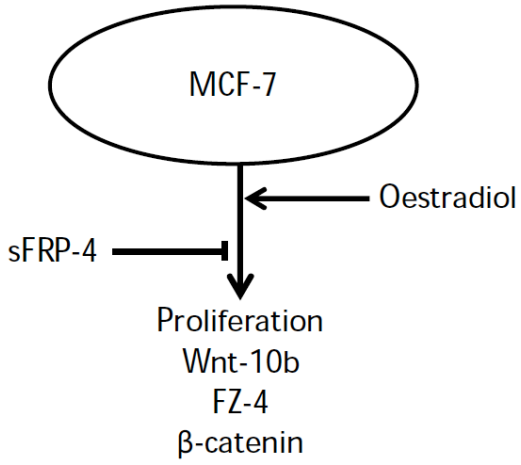


Figure 5: Oestradiol increases MCF-7 cellular proliferation and the expression of Wnt-10b, FZ-4, and β-catenin at the gene level. However, sFRP-4 is able to block these oestradiol-induced effects.

When oestradiol and sFRP-4 were combined there was a significant reduction in the anticipated effect of oestradiol-induced cell growth in the MCF-7 cells. As there was no significant increase in active β-catenin protein due to oestradiol treatment alone, it was anticipated that when sFRP-4 was combined with oestradiol, it should still reduce the amount of β-catenin, as had been seen with sFRP-4 treatment alone; and this would be consistent with sFRP-4 acting through the Wnt signalling pathway. However, this was

not the case. It is possible that, because the Wnt signalling pathway results in transcriptional regulation of genes that are also transcriptionally activated by oestradiol, there is some overlap of these two pathways. That is, when sFRP-4 down regulates the Wnt pathway, the consequent absence of the TCF/LEF transcription factors results in a less potent response to oestradiol-induced gene expression of cell cycle regulatory genes. This then diminishes oestradiol-induced cell growth.

No change was detected in sFRP-4 mRNA or protein in any of the treatments, suggesting that oestradiol itself does not regulate the expression of sFRP-4 in the MCF-7 cell line. It is not presently known what does increase sFRP-4 expression in normal cells, although it has been associated with apoptosis [23]. More recently, silencing of sFRP-4 *via* promoter methylation has been demonstrated in β-catenin deficient mesothelioma cell lines [23]. Re-expression of sFRP-4 in these cells has been shown to not only block Wnt signalling, but to induce apoptosis as well as suppress cell growth [24]; further supporting a growth suppressant and apoptotic role for sFRP-4.

Membranous sFRP-4 expression in prostate cancer has been shown to be predictive of good prognosis, and transfection of sFRP-4 into prostate cancer cells reduces cell proliferation [6]. Our data indicate that sFRP-4 is able to suppress the growth of breast cancer cells and that it may also suppress oestradiol-stimulated growth. It would be valuable to determine sFRP-4 expression in a cohort of breast cancer samples, together with other known markers such as Estrogen Receptor status, to ascertain whether sFRP-4 is also associated with a favourable prognosis in breast cancer.

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