The Wnt regulator SFRP4 inhibits mesothelioma cell proliferation, migration, and antagonizes Wnt3a via its netrin-like domain

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Abstract. Secreted frizzled related proteins (SFRPs) are a family of Wnt regulators which are frequently downregulated in cancers. In malignant mesothelioma (MM), downregulation of SFRP4 has been reported as a mechanism which contributes to aberrant activation of oncogenic Wnt signaling. Here we investigated the biological consequences of SFRP4 in two mesothelioma cell models where this protein is downregulated. We used recombinant SFRP4 and transient overexpression to study changes in proliferation, migration and downstream signaling. We found that recombinant SFRP4 inhibited both proliferation and migration of MM cells as well as abrogating the stimulatory effect of recombinant Wnt3a. Morphologically SFRP4 induced a cytotoxic effect distinct from apoptosis and consistent with mitotic catastrophe. Overexpression of SFRP4 in these cell lines displayed similar effects as endogenous protein on cell viability, migration and nuclear morphology. We also used expression constructs to examine the role of the SFRP4 cysteine rich domain (CRD) and a netrin-like domain (NLD) in these effects. Interestingly, we found it was the NLD which mediated the biological effects of SFRP4 in these cells. Our results indicate that SFRP4 inhibits mesothelioma proliferation, migration and activates alternative cell death pathways. The finding that the NLD is responsible for these has broader implications for this protein family. Overall this study suggests that the Wnt pathway may prove a promising target for therapy in mesothelioma.

Introduction

Malignant pleural mesothelioma is an aggressive type of tumor which is invariably fatal in outcome and is associated with past asbestos exposure. While mesothelioma is a rare tumor it continues to have an increased incidence due to the previous widespread use of asbestos products in industrial nations and continuing use in some developing countries (1). Understanding the alterations in molecular signaling that occur in mesotheliomas can help not just to develop suitable biomarkers but also lead to new targeted therapies. Many pathways have been identified as potential targets in mesothelioma by studying molecular changes in patient tumor samples (2,3). One pathway which has been identified as a potentially significant pathway in mesothelioma is the Wnt signaling pathway (4-6).

Aberrant activation of Wnt signaling to drive proliferation, survival and invasion has been frequently described in cancer (reviewed in ref. 7). One mechanism which has been described is the downregulation of members of a family of regulatory proteins: the secreted frizzled related proteins (SFRP) (8). Studies of SFRP family expression and function in mesothelioma cells and tumors by other laboratories (9-11) and our own (6) have shown that one member of this family, SFRP4, is differentially downregulated in this cancer. There is evidence that downregulation of SFRP4 by promoter methylation could result in Wnt pathway activation in mesothelioma (10). Previous mechanistic studies showing induction of apoptosis and growth inhibition in response to SFRP4 have used a mesothelioma cell model which was β-catenin deficient and canonical signaling was not present (10). Most mesothelioma tumors and cell lines have been found to express β-catenin (11-13). The SFRPs consist of two domains, a Fz-like cysteine rich domain (CRD) and a netrin-like domain (NLD). Because of its structural similarity to the extracellular domain of the Fz receptors the SFRP was initially believed to play a role analogous in binding to Wnt proteins (14), however, more recent studies have also implicated the NLD (15,16). Since SFRP4 is the most commonly downregulated SFRP in mesothelioma (6,9-11) we undertook to investigate the effect of SFRP4 and its domains in more relevant β-catenin expressing mesothelioma models which are capable of canonical Wnt signaling and which express low or negligible native SFRP4.

In this study we investigate the effects of both exogenous and endogenous overexpression of SFRP4 upon proliferation, migration, cell behaviour and downstream signaling. We

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report that re-introduction of SFRP4 to mesothelioma cells inhibits cell proliferation, migration and induces a non-apoptotic cell death programme. Since SFRP4 expression is widely downregulated in mesothelioma (9,11) these findings support the targeting of Wnt signaling in mesothelioma at the level of upstream regulators as a potential therapeutic approach. Furthermore, we found that the effects of SFRP4 were mediated by the NLD providing further insight into the biology of these important Wnt regulators.

Materials and methods

Cell culture and reagents. The malignant mesothelioma cell lines JU77 and ONE58 were used in this study. These cell lines were originally derived from pleural effusions of different patients presenting with malignant pleural mesothelioma (17). All cells were cultured and maintained in medium R5, which is RPMI-1640 plus 5% heat-inactivated fetal bovine serum (FBS), 300 mM L-glutamine, 120 μg/ml penicillin and 100 μg/ml streptomycin (all from Thermo Fisher, Hyclone, VIC, Australia). All cell cultures were grown at 37˚C in a 5% CO_2 humidified atmosphere. Recombinant human proteins Wnt3a and SFRP4 were obtained from R&D Systems (MN, USA). Cisplatin (Oncotain) was obtained from Mayne Pharma (VIC, Australia).

MTT assay. Cell viability in response to various treatments was quantitated by an MTT assay. Cells were seeded into 96-well plates at a density of 10,000-20,000 cells/well, depending on the experiment. Following 24-h incubation treatments were added and cells incubated for a further 24-72 h (depending on experiments) then the MTT assay was performed as previously described (18) and absorbance was read at 595 nm with a microplate reader (Enspire, Perkin-Elmer). For each treatment, the mean absorbance for the replicates was calculated. The control samples were untreated cells or cells treated with vehicle alone and control data was set to 100% viable cells and all other data were normalised to this value based on absorbance value. The data were expressed as mean ± standard deviation.

Proliferation. Cells were seeded at a density of $5x10^4$ cells/well in 24-well plates and treatments added at 24 h. The cells were harvested 48 h later by trypsinization and evaluated by a trypan blue assay using a Countess Automated Cell Counter (Life Technologies).

Cell migration assay. A scratch wound assay was used to assess cell migration. Cells were seeded in 6-well plates at a density of $3x10^5$ cells/well and grown to confluency. The monolayers were scratched using a 10-μl pipette tip to produce 2 crosses per well and washed with PBS to remove any non-adherent cells and debris. The field of view was kept constant by drawing a line on the well bottom perpendicular to the centre of T scratch site and using this as a reference point. The monolayers were imaged centred on the crosses using a Canon EOS digital camera mounted on an Olympus CK2 microscope after 0 and 6 h. The open area of the wound was quantitated by automatic image analysis using TScratch software (19) with the default parameter settings.

Assessment of mitochondrial membrane potential. Mitochondrial outer membrane potential changes were analysed using the cationic dye JC-1 (5, 5', 6, 6'-tetrachloro-1, 1', 3, 3'-tetraethyl-benzimidazolcarbocyanine iodide) essentially as previously described (20). Data are presented as the ratio of red to green fluorescence intensity and mitochondrial depolarization is indicated by a decrease in this parameter.

Caspase-3 activity assay. Activation of effector caspases was determined using the Caspase-3 Assay Kit#2 (Molecular Probes, USA) with a modified protocol as previously described (20).

Immunoblot analysis. Cell monolayers were washed once with PBS and lysed by addition of 1X SDS loading buffer (Bio-Rad) containing 5% mercaptoethanol (Sigma-Aldrich). The lysate was disrupted by pipetting and transferred to a microfuge tube on ice. Samples were sonicated (QSonica, Q125, CT, USA) at amplitude 40, 3x 15 sec at 30-sec intervals then centrifuged at 10,000 x g for 5 min and the supernatants recovered and stored at -20˚C. Proteins were resolved on 4-12% Mini Protean TGX gels (Bio-Rad) and transferred to nitrocellulose membrane (Bio-Rad) then probed with the following antibodies: monoclonal rabbit anti-human β-catenin [Cell Signaling Technology (CST), MA, USA], polyclonal rabbit anti-Dvl-3 (CST) and monoclonal mouse anti-β-actin (Sigma-Aldrich). After incubation with horseradish peroxidase conjugated secondary antibody (Jackson Immunosearch, PA, USA) chemiluminescent detection was performed with Clarity ECL substrate (Bio-Rad) and images captured using a GelDoc XR imaging system (Bio-Rad).

Live cell imaging for nuclear morphology. At the completion of the experiment the media was aspirated and cells stained with 1 μg/ml Hoechst 33342 solution (Thermo Scientific) for 5 min. The cells were washed with PBS and fresh media added before imaging with an Olympus IX-51 inverted fluorescent microscope.

Plasmid constructs and transfection. Expression vectors for the human SFRP4 gene and the CRD and NLD domains were a kind gift from Professor Robert Friis, University of Bern, Switzerland. The SFRP4 constructs were prepared by PCR and cloning into the pEGFP-N1 vector (Clontech, CA, USA) so that the full length SFRP4, the CRD domain or the NLD C terminal domain were expressed in frame as amino terminal fusions to the GFP. The SFRP4, CRD and NLD constructs retained the Kozak and signal sequences of SFRP4. The parental pEGFP-N1 vector expressing GFP was used as a control in all transfection experiments although mock transfected and untreated cultures were routinely included as assay controls. Plasmid DNA for transfection was prepared using a HiSpeed Plasmid Midi kit (Qiagen, VIC, Australia). Transient transfections were performed using FuGENE® HD reagent and the pEGFP-N1 plasmid vector constructs. Following transfection with reagent:DNA in a ratio of 3:1 for 24 h, the transfection reagent was removed from the cells and replaced with standard complete RPMI medium. Transfection efficiency was assessed by fluorescence microscopy.

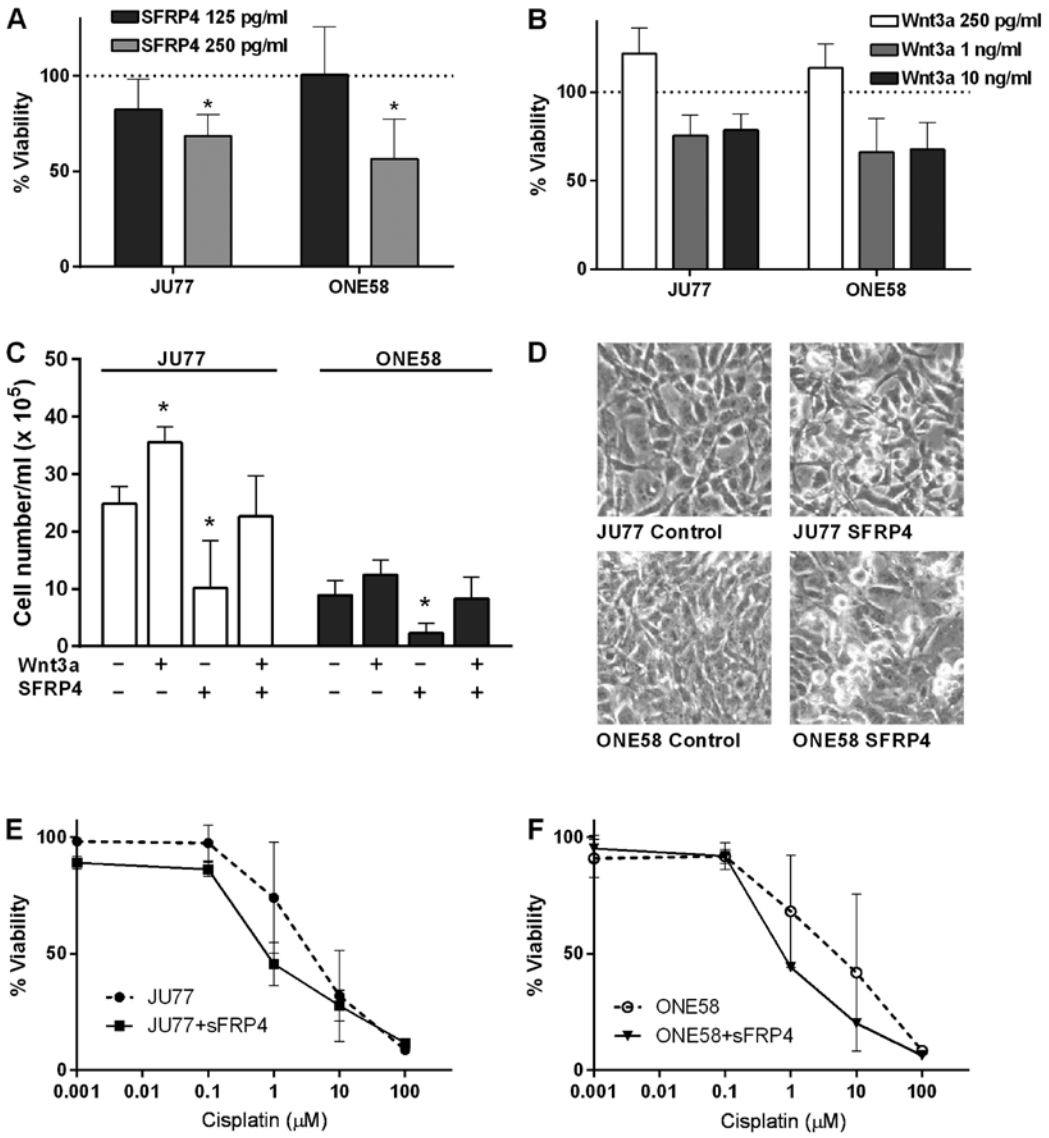

Figure 1. SFRP4 modulates proliferation, survival and morphology in mesothelioma cells. Mesothelioma cells were treated for 24 h with recombinant (A) SFRP4 or (B) Wnt3a and proliferation/viability measured by MTT assay. Data are normalized relative to control (100% - dotted line). (C) Cultures were treated with 250 pg/ml recombinant SFRP4 and/or Wnt3a for 48 h and proliferation/viability determined by trypan blue assay. (D) Photomicrographs of cultures treated with 250 pg/ml recombinant SFRP4 for 48 h. (E) JU77 or (F) ONE58 cells were treated with cisplatin (0-100 μM) and 250 pg/ml recombinant SFRP4 for 48 h and cytotoxicity measured by MTT assay. All data shown are mean ± SD of 3 independent experiments ($^*p<0.05$ relative to control).

Statistical analysis. Statistical comparison between two groups was performed using unpaired t-test with Prism v6.07 (GraphPad software). The difference was determined to be statistically significant at $p<0.05$. Determination and statistical comparison of IC_{50} was performed by non-linear regression analysis and F-test using Prism v6.07.

Results

Exogenous SFRP4 downregulates proliferation and enhances chemosensitivity in mesothelioma cells. We have previously reported that SFRP4 conditioned media downregulated mesothelioma cell proliferation (6) and here we conducted experiments to examine the effects of purified recombinant SFRP4. We found that recombinant SFRP4 caused dose-dependent inhibition of cell viability/proliferation in both mesothelioma cell lines examined as determined by MTT assay (Fig. 1A). The effect of Wnt3a was also determined in a similar experiment and interestingly it was found that while 250 pg/ml recombinant Wnt3a did appear to upregulate proliferation, at higher concentrations there was an inhibitory effect (Fig. 1B) although these effects were not statistically significant. To further confirm these results we next examined whether SFRP4 could influence the effects of recombinant Wnt3a upon mesothelioma cell proliferation using a trypan blue assay. These experiments showed that 250 pg/ml SFRP4 significantly inhibited proliferation in both JU77 and ONE58 and this effect was blocked by 250 pg/ml Wnt3a (Fig. 1C). Microscopic examination of cultures treated with SFRP4 demonstrated significant morphological effects in both cell lines. Many cells demonstrated an apparent cytopathic effect with a characteristic loss of attachment and rounding up appearance after SFRP4 treatment (Fig. 1D). We next examined whether SFRP4 influenced the response of mesothe-

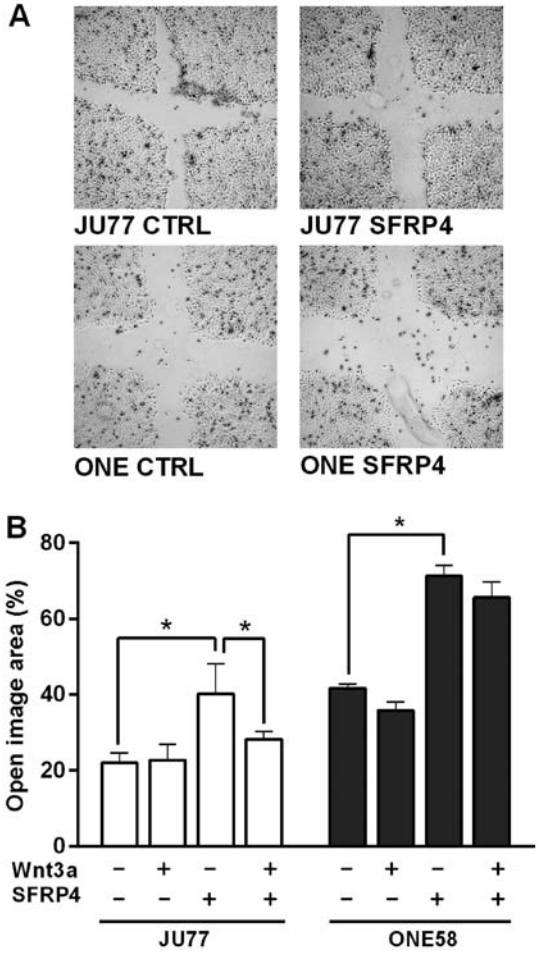

Figure 2. Effect of recombinant Wnt3a and sFRP4 on mesothelioma cell migration. (A) Representative images of effect of recombinant SFRP4 (250 pg/ml) upon mesothelioma migration by scratch assay. (B) Quantitative analysis of cell migration in response to recombinant SFRP4 (250 pg/ml) and/or Wnt3a (250 pg/ml) by scratch assay. All results are mean ± SD of at least 3 independent experiments, $^{*}p<0.05$.

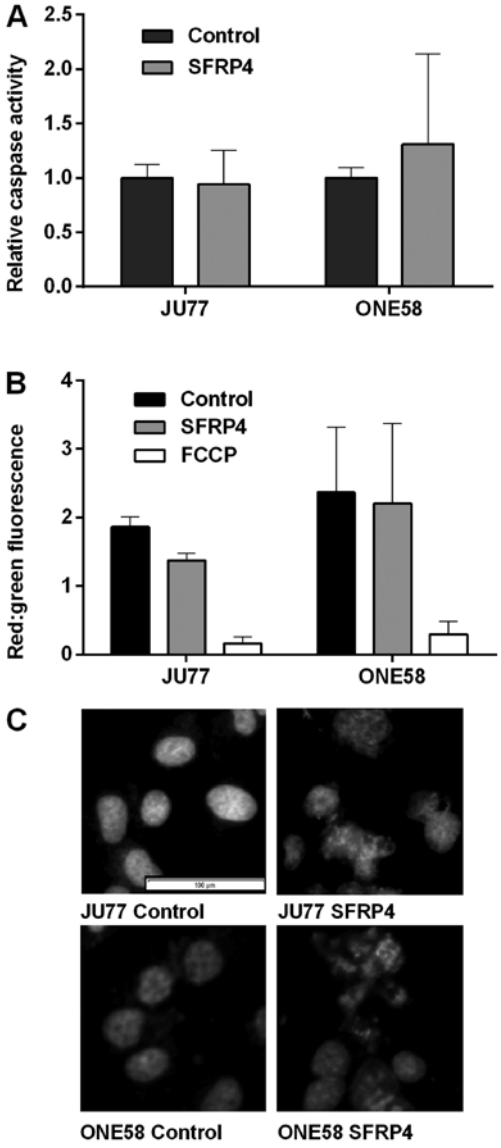

Figure 3. Mechanisms of cell death in SFRP4 treated mesothelioma cells. (A) Caspase-3/7 activation and (B) mitochondrial depolarization (determined by JC-1 assay) were not observed in cells treated with 250 pg/ml recombinant SFRP4 for 24 h. (C) Changes in nuclear morphology after SFRP4 (250 pg/ml) treatment for 48 h as determined by Hoechst 33258 staining showing multi-nucleated cells and chromosomal vesicles.

lioma cells to cytotoxic drug insults. Cells were treated with 0.001-100 μM cisplatin for 48 h in the presence of 250 pg/ml recombinant SFRP4 and assayed for cell proliferation. In both JU77 and ONE58 cells SFRP4 did appear to sensitize mesothelioma cells to cisplatin (Fig. 1E and F) with a statistically significant difference in the IC_{50} values as determined by non-linear regression (p=0.0233 and 0.0046 respectively).

SFRP4 inhibits mesothelioma cell migration. Previous studies have suggested that SFRPs can affect migration in cancer cells (21). Therefore the effect of SFRP4 and Wnt3a on the migration of malignant mesothelioma cells was investigated using a scratch or wound healing assay. In both cell lines SFRP4 appeared to inhibit wound closure (Fig. 2A) and this result was significant as confirmed by open wound area image analysis (Fig. 2B). Interestingly, Wnt3a had little effect upon wound closure in JU77 and ONE58 cells but significantly ameliorated the effect of SFRP4 in JU77 cells.

Mechanisms of SFRP4 induces cell death in mesothelioma cells. To examine in more detail the cytopathic effect of SFRP4 observed in cell viability and morphological assays we endeavored to characterize cell death mechanisms in response to SFRP4. In JU77 and ONE58 cells SFRP4 did not have a significant effect upon caspase-3 activation (Fig. 3A). An important event in apoptotic signaling is disruption of the mitochondrial membrane and loss of the outer membrane potential (OMP). Only JU77 cells showed a modest decline in OMP with SFRP4 treatment, however, this was not statistically significant and did not approach the level of the positive control decoupling agent FCCP (Fig. 3B)

Cells undergoing apoptosis show characteristic changes in nuclear morphology with chromatin condensation and nuclear fragmentation. The nuclear morphology of JU77 and ONE58 cells was observed by Hoechst 33342 staining after 48 h of treatment (Fig. 3C). Interestingly, in cells treated with SFRP4 characteristic apoptotic changes were not seen although there were distinctive changes in nuclear morphology. These

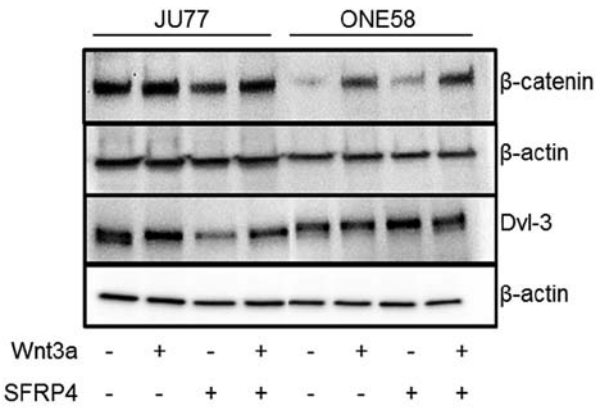

Figure 4. Downstream signaling in response to SFRP4 and Wnt3a. Cells were treated with 250 pg/ml of recombinant protein for 6 h and assayed by western blotting using appropriate antibodies. Results are representative of three different determinations.

changes of multinucleated cells with chromosome vesicles were characteristic of mitotic catastrophe (22). These changes were consistently observed in both JU77 and ONE58 cells in response to SFRP4. Apoptotic nuclei were not observed.

Effect of SFRP4 upon downstream signaling. To further understand the effects of SFRP4 upon mesothelioma cells β-catenin and Dvl-3 were assayed by western blotting. In both JU77 and ONE58 Wnt3a treatment upregulated β-catenin levels (Fig. 4), while in JU77 SFRP4 downregulated β-catenin this was not observed in ONE58 although basal β-catenin levels were quite low in this cell line. Similarly, in JU77, Wnt3a induced apparent Dvl-3 phosphorylation while SFRP4 downregulated Dvl-3. Surprisingly, these effects were not seen in ONE58 despite other assays showing similar responses to SFRP4 in both cell lines.

Effect of endogenous overexpression of SFRP4 and domains upon mesothelioma cell proliferation and morphology. Having established that recombinant SFRP4 had effects upon mesothelioma cell proliferation, viability and migration, we next examined the effect of endogenous overexpression of the full length protein as well as its domains: the NLD and CRD. Previous studies have reported conflicting evidence regarding the role of different SFRP domains in Wnt signaling regulation and we explored this aspect. Following 48 h of transfection the observed effects were quite small by microscopic examination in comparison to exogenous treatment with SFRP4 (data not shown). However, at 6 days the effects upon cell morphology of the SFRP4 and NLD constructs were similar to those seen with recombinant SFRP4 (Fig. 1D). In both cell lines overexpression of SFRP4 and the NLD significantly downregulated proliferation/viability (Fig. 5A). The CRD had little effect in either cell line. These results were consistent with the effect

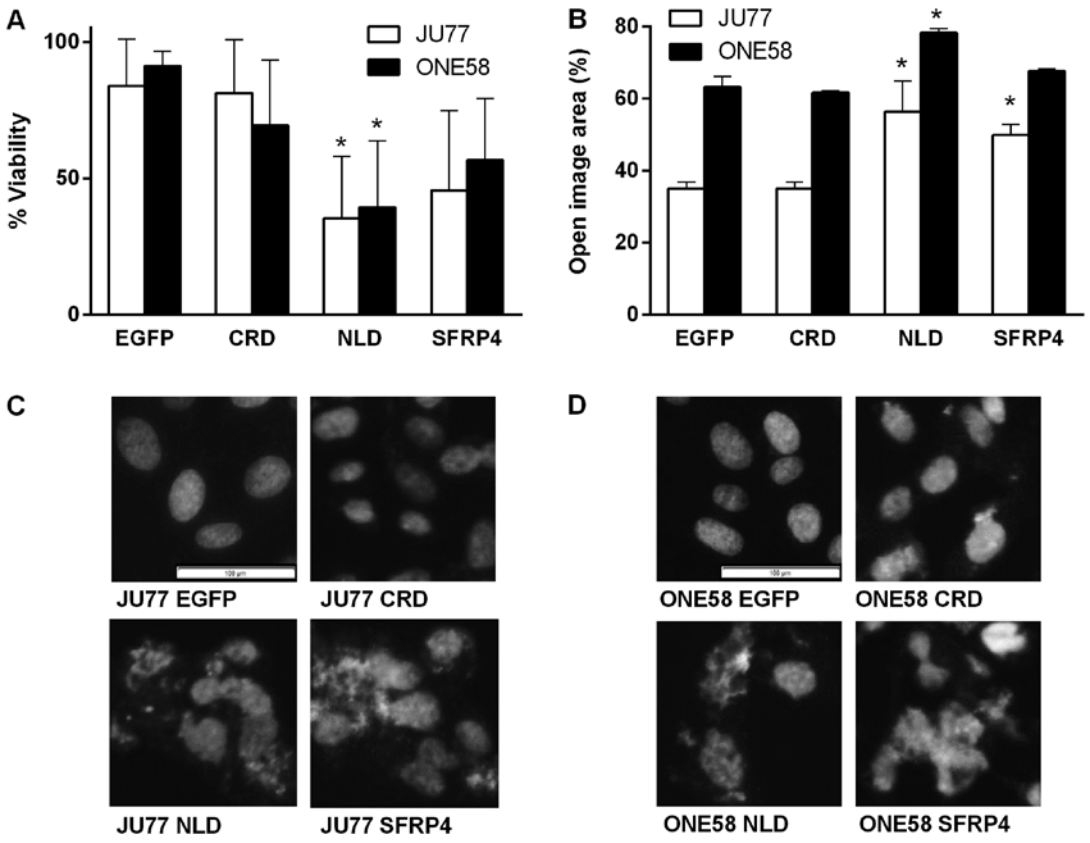

Figure 5. Effect of SFRP4 and domain overexpression upon JU77 and ONE58 cells. (A) Cell viability was determined by MTT assay 48 h after transfection with the relevant construct. (B) Migration was assayed 48 h after transfection by scratch assay and quantitated using Tscratch software. Changes in nuclear morphology of (C) JU77 and (D) ONE58 after transfection with relevant constructs for 48 h as determined by Hoechst 33258 staining. All data shown are mean ± SD of 3 independent experiments ($^{*}p<0.05$ relative to control). Images are representative of 3 independent experiments.

of recombinant SFRP4 and demonstrated the NLD alone was sufficient for the cell viability effects of SFRP4.

Effect of endogenous SFRP4 and domain expression on mesothelioma cell migration. In order to further investigate the effects of endogenous SFRP4 and domains upon mesothelioma cells transfectants were investigated using a wound healing assay. The effects of SFRP4 overexpression were not as great as that seen with the endogenous protein but it was still apparent in both cell lines. Quantitation of these effects showed that SFRP4 and the NLD significantly inhibited wound closure while the CRD had no effect (Fig. 5B). These results are consistent with the effect of recombinant SFRP4 and also demonstrate that as for proliferation the NLD mediates the major effects of SFRP4 upon mesothelioma cells.

Overexpression of SFRP4 and its NLD induce nuclear morphological changes consistent with mitotic catastrophe. We next examined whether SFRP4 and domain overexpression elicited similar effects upon nuclear morphology as those seen with exogenous recombinant protein. We did in fact observe that in both JU77 and ONE58 cell expression of SFRP4 and the NLD induced a nuclear morphology characteristic of mitotic catastrophe which was consistent with that shown in Fig. 3 (Fig. 5C and D). Cells expressing EGFP alone or the CRD did not display this effect (Fig. 5C and D).

Discussion

The experiments described in this study were initiated based upon findings by other laboratories (9-11) and our own study (6) showing differential downregulation of SFRP4 in mesothelioma cells and tissues. Evidence from the literature has demonstrated that most mesotheliomas are β-catenin-positive (4,12,13). Therefore it was of great interest to us to investigate the biological effects and downstream signaling of SFRP4 in β-catenin expressing mesothelioma cell models. We established that both exogenous recombinant SFRP4 and endogenous SFRP4 overexpression had similar effects resulting in downregulation of proliferation and migration. Furthermore, a novel finding of our study was that SFRP4 induced a cytopathic effect that was apparently distinct from apoptosis and consistent with mitotic catastrophe. Notably, we were also able to provide evidence enhancing our understanding of the biology of SFRPs since we found that the effects of SFRP4 upon mesothelioma cells were largely mediated by the netrin-like domain.

Overall the findings of our study are consistent with downregulation of SFRP4 playing a role in the dysregulation of Wnt signaling which promotes mesothelioma pathogenesis. These findings broadly agree with those reported in other cancers where SFRP4 has been reported to act as a tumor suppressor (23-25) although there are conflicting reports in some cancers (26-28). We found that SFRP4 both downregulated mesothelioma proliferation and antagonized the effects of Wnt3a alone. Interestingly, the dose response study of Wnt3a revealed a biphasic response in proliferation. These effects may be due to feedback regulation of the pathway at high Wnt concentrations leading to downregulation of β-catenin levels (29). A key finding of this study was that the cytopathic effect of SFRP4 upon mesothelioma cells occurred via alternative pathways characteristic of mitotic catastrophe (22) and this contrasts with literature reports of SFRP4 inducing apoptosis in other cell types (10,30,31). This observation was confirmed by SFRP4 overexpression experiments and while it has been reported that ultimately mitotic catastrophe can trigger apoptosis we did not observe it here (22).

Wnt signaling is known to target genes involved in cell migration (7), however, most studies which have investigated the role of SFRP4 in cancer have focused upon proliferation or apoptosis although SFRP4 has been shown to regulate migration of endothelial and ovarian cancer cells (24,32). We found that migration of both cell lines was greatly inhibited by SFRP4. We know from a previous study that these mesothelioma cells express Wnt2b, Wnt3 and Wnt4 and Wnt5a although Wnt2b and Wnt4 are downregulated (6). Hence, endogenously expressed Wnt3 and Wnt5a are possible SFRP4 interacting partners which may mediate this effect. The fact that Wnt3a alone had little effect upon migration but did reduce the effect of SFRP4 in JU77 cells suggests that the target of SFRP4 interaction to influence migration may be subject to competition by Wnt3a in these cells but not in ONE58. It has recently been reported that SFRP4 does not bind with Wnt3a or inhibit Wnt3a signaling (33). This is inconsistent with our results which suggest that there is some interaction between SFRP4 and Wnt3a and that SFRP4 can antagonize the effects of Wnt3a. These discrepancies emphasize the context dependency of Wnt signaling since SFRPs may also act by interactions with Fz receptors and may not need to directly bind Wnts (34). Furthermore, this supports some mechanistic differences between the two cell lines despite the phenotypic effects of SFRP4 being broadly similar. This was most frequently seen in experiments using ONE58 where Wnt3a had a less pronounced phenotypic effect (Figs. 1C and 2B) although it clearly induced β-catenin accumulation which was more easily observed in ONE58 (Fig. 4). These results suggest that basal β-catenin levels are higher in JU77 so that it was more difficult to discern the effect of Wnt3a. These phenotypic differences in response to Wnt3a may reflect the different repertoire of Fz receptors expressed in these two cell lines (6).

Overall it was found that endogenous SFRP4 expression exerted similar effects on proliferation, migration and nuclear morphology to those found using exogenous protein. The key finding here was that the effects of SFRP4 were also seen when cells were transfected with NLD expression constructs but not in cells overexpressing the CRD domain. This is significant in the context of our understanding of SFRPs since it is consistent with recent studies that show that the NLD of other SFRPs bind with Wnts at high affinity and can antagonize Wnt signaling (15,16,34) while the CRD most likely acts through interactions with Fz receptors (34).

In conclusion, SFRP4 which is downregulated in mesothelioma is able to inhibit proliferation, migration and induce nuclear changes characteristic of mitotic catastrophe when reintroduced to mesothelioma cells using recombinant protein or overexpression. A key finding of our study is that these effects are mainly caused by the functions of the SFRP4 netrin-like domain and the CRD has limited effect in these models. SFRP4 is also able to inhibit signaling by Wnt3a in mesothelioma cells. Overall the data provide supporting

evidence for the targeting of the Wnt pathway in mesothelioma and new evidence regarding the biology of SFRP4 which is likely to have relevance to other members of this family and their role in regulation of Wnt signaling and tumor biology.

Acknowledgements

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References

1. van Meerbeeck JP, Scherpereel A, Surmont VF and Baas P: Malignant pleural mesothelioma: The standard of care and challenges for future management. Crit Rev Oncol Hematol 78: 92-111, 2011.
2. Zucali PA, Ceresoli GL, De Vincenzo F, Simonelli M, Lorenzi E, Gianoncelli L and Santoro A: Advances in the biology of malignant pleural mesothelioma. Cancer Treat Rev 37: 543-558, 2011.
3. Rascoe PA, Jupiter D, Cao X, Littlejohn JE and Smythe WR: Molecular pathogenesis of malignant mesothelioma. Expert Rev Mol Med 14: e12, 2012.
4. Uematsu K, Kanazawa S, You L, He B, Xu Z, Li K, Peterlin BM, McCormick F and Jablons DM: Wnt pathway activation in mesothelioma: Evidence of Dishevelled overexpression and transcriptional activity of beta-catenin. Cancer Res 63: 4547-4551, 2003.
5. Uematsu K, Seki N, Seto T, Isoe C, Tsukamoto H, Mikami I, You L, He B, Xu Z, Jablons DM, *et al*: Targeting the Wnt signaling pathway with dishevelled and cisplatin synergistically suppresses mesothelioma cell growth. Anticancer Res 27B: 4239-4242, 2007.
6. Fox SA, Richards AK, Kusumah I, Perumal V, Bolitho EM, Mutsaers SE and Dharmarajan AM: Expression profile and function of Wnt signaling mechanisms in malignant mesothelioma cells. Biochem Biophys Res Commun 440: 82-87, 2013.
7. Anastas JN and Moon RT: WNT signalling pathways as therapeutic targets in cancer. Nat Rev Cancer 13: 11-26, 2013.
8. Surana R, Sikka S, Cai W, Shin EM, Warrier SR, Tan HJ, Arfuso F, Fox SA, Dharmarajan AM and Kumar AP: Secreted frizzled related proteins: Implications in cancers. Biochim Biophys Acta 1845: 53-65, 2014.
9. Lee AY, He B, You L, Dadfarmay S, Xu Z, Mazieres J, Mikami I, McCormick F and Jablons DM: Expression of the secreted frizzled-related protein gene family is downregulated in human mesothelioma. Oncogene 23: 6672-6676, 2004.
10. He B, Lee AY, Dadfarmay S, You L, Xu Z, Reguart N, Mazieres J, Mikami I, McCormick F and Jablons DM: Secreted frizzled-related protein 4 is silenced by hypermethylation and induces apoptosis in beta-catenin-deficient human mesothelioma cells. Cancer Res 65: 743-748, 2005.
11. Kohno H, Amatya VJ, Takeshima Y, Kushitani K, Hattori N, Kohno N and Inai K: Aberrant promoter methylation of WIF-1 and SFRP1, 2, 4 genes in mesothelioma. Oncol Rep 24: 423-431, 2010.
12. Abutaily AS, Collins JE and Roche WR: Cadherins, catenins and APC in pleural malignant mesothelioma. J Pathol 201: 355-362, 2003.
13. Orecchia S, Schillaci F, Salvio M, Libener R and Betta P-G: Aberrant E-cadherin and gamma-catenin expression in malignant mesothelioma and its diagnostic and biological relevance. Lung Cancer 45 (Suppl 1): S37-S43, 2004.
14. Lin K, Wang S, Julius MA, Kitajewski J, Moos M Jr and Luyten FP: The cysteine-rich frizzled domain of Frzb-1 is required and sufficient for modulation of Wnt signaling. Proc Natl Acad Sci USA 94: 11196-11200, 1997.
15. Bhat RA, Stauffer B, Komm BS and Bodine PVN: Structure-function analysis of secreted frizzled-related protein-1 for its Wnt antagonist function. J Cell Biochem 102: 1519-1528, 2007.
16. Lopez-Rios J, Esteve P, Ruiz JM and Bovolenta P: The Netrin-related domain of Sfrp1 interacts with Wnt ligands and antagonizes their activity in the anterior neural plate. Neural Dev 3: 19, 2008.
17. Manning LS, Whitaker D, Murch AR, Garlepp MJ, Davis MR, Musk AW and Robinson BW: Establishment and characterization of five human malignant mesothelioma cell lines derived from pleural effusions. Int J Cancer 47: 285-290, 1991.
18. Whittell LR, Batty KT, Wong RPM, Bolitho EM, Fox SA, Davis TME and Murray PE: Synthesis and antimalarial evaluation of novel isocryptolepine derivatives. Bioorg Med Chem 19: 7519-7525, 2011.
19. Gebäck T, Schulz MMP, Koumoutsakos P and Detmar M: TScratch: A novel and simple software tool for automated analysis of monolayer wound healing assays. Biotechniques 46: 265-274, 2009.
20. Cregan IL, Dharmarajan AM and Fox SA: Mechanisms of cisplatin-induced cell death in malignant mesothelioma cells: Role of inhibitor of apoptosis proteins (IAPs) and caspases. Int J Oncol 42: 444-452, 2013.
21. Roth W, Wild-Bode C, Platten M, Grimmel C, Melkonyan HS, Dichgans J and Weller M: Secreted Frizzled-related proteins inhibit motility and promote growth of human malignant glioma cells. Oncogene 19: 4210-4220, 2000.
22. Vakifahmetoglu H, Olsson M and Zhivotovsky B: Death through a tragedy: Mitotic catastrophe. Cell Death Differ 15: 1153-1162, 2008.
23. Horvath LG, Lelliott JE, Kench JG, Lee CS, Williams ED, Saunders DN, Grygiel JJ, Sutherland RL and Henshall SM: Secreted frizzled-related protein 4 inhibits proliferation and metastatic potential in prostate cancer. Prostate 67: 1081-1090, 2007.
24. Ford CE, Jary E, Ma SSQ, Nixdorf S, Heinzelmann-Schwarz VA and Ward RL: The Wnt gatekeeper SFRP4 modulates EMT, cell migration and downstream Wnt signalling in serous ovarian cancer cells. PLoS One 8: e54362, 2013.
25. Warrier S, Balu SK, Kumar AP, Millward M and Dharmarajan A: Wnt antagonist, secreted frizzled-related protein 4 (sFRP4), increases chemotherapeutic response of glioma stem-like cells. Oncol Res 21: 93-102, 2013.
26. Turashvili G, Bouchal J, Burkadze G and Kolar Z: Wnt signaling pathway in mammary gland development and carcinogenesis. Pathobiology 73: 213-223, 2006.
27. Huang D, Yu B, Deng Y, Sheng W, Peng Z, Qin W and Du X: SFRP4 was overexpressed in colorectal carcinoma. J Cancer Res Clin Oncol 136: 395-401, 2010.
28. Mortensen MM, Høyer S, Lynnerup A-S, Ørntoft TF, Sørensen KD, Borre M and Dyrskjøt L: Expression profiling of prostate cancer tissue delineates genes associated with recurrence after prostatectomy. Sci Rep 5: 16018, 2015.
29. Lustig B, Jerchow B, Sachs M, Weiler S, Pietsch T, Karsten U, van de Wetering M, Clevers H, Schlag PM, Birchmeier W and Behrens J: Negative feedback loop of Wnt signaling through upregulation of conductin/axin2 in colorectal and liver tumors. Mol Cell Biol 22: 1184-1193, 2002.
30. Drake JM, Friis RR and Dharmarajan AM: The role of sFRP4, a secreted frizzled-related protein, in ovulation. Apoptosis 8: 389-397, 2003.
31. Maganga R, Giles N, Adcroft K, Unni A, Keeney D, Wood F, Fear M and Dharmarajan A: Secreted Frizzled related protein-4 (sFRP4) promotes epidermal differentiation and apoptosis. Biochem Biophys Res Commun 377: 606-611, 2008.
32. Muley A, Majumder S, Kolluru GK, Parkinson S, Viola H, Hool L, Arfuso F, Ganss R, Dharmarajan A and Chatterjee S: Secreted frizzled-related protein 4: An angiogenesis inhibitor. Am J Pathol 176: 1505-1516, 2010.
33. Carmon KS and Loose DS: Development of a bioassay for detection of Wnt-binding affinities for individual frizzled receptors. Anal Biochem 401: 288-294, 2010.
34. Rodriguez J, Esteve P, Weinl C, Ruiz JM, Fermin Y, Trousse F, Dwivedy A, Holt C and Bovolenta P: SFRP1 regulates the growth of retinal ganglion cell axons through the Fz2 receptor. Nat Neurosci 8: 1301-1309, 2005.