Short Report

Loss of hSef promotes metastasis through upregulation of EMT in prostate cancer

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We have previously reported that the negative signaling regulator Similar Expression to FGF (hSef) is downregulated in prostate cancer and its loss is associated with clinical metastasis. Here, we explored the mechanistic basis of this finding. We first confirmed our clinical observation by testing hSef manipulation in an in vivo metastasis model. hSef stable expressing cells (PC3M-hSef) or empty vector controls (PC3M-EV) were injected subcutaneously into the lateral thoracic walls of NOD-SCID gamma mice and lungs were harvested at autopsy. In this model, 6/7 PC3M-EV xenografts had definitive lung micro-metastasis whilst only 1/6 PC3M-hSef xenografts exhibited metastasis recapitulating the clinical scenario (p = 0.03). Gene expression studies revealed key perturbations in genes involved in cell motility and epithelial to mesenchymal transition (EMT) along with alterations in cognate signaling pathways. These results were validated in an EMT specific PCR array whereby hSef over-expression and silencing reciprocally altered E-Cadherin expression (p = <0.001) amongst other EMT markers. Immunohistochemistry of excised tumors from the xenografts also confirmed the effect of hSef in suppressing E-Cadherin expression at the protein level. Phosphokinase arrays further demonstrated a role for hSef in attenuating signaling of not only ERK-MAPK but also the JNK and p38 pathways as well. Taken together, these data suggest evidence that loss of hSef may be a critical event facilitating tumor dissemination of prostate cancer through alteration of EMT. Detection of downregulated hSef, along with other negative regulators, may therefore be a useful biomarker heralding a transition to a metastatic phenotype and warrants further exploration in this context.

Aberrant intracellular signaling is considered one of the hallmarks of cancer.1,2 Intracellular signaling however is subject to different levels of regulation that serve to attenuate the eventual impact of stimulation.3 Negative signaling regulators (NSR) are feedback-induced mechanisms that are innate to the cell. There is now emerging evidence that NSR are themselves altered in the transition from benign to malignant cells.4 Similar Expression to FGF (Sef) has been particularly well characterized as a tumor suppressor in a diverse range of cancers.5

The first of the Sef proteins to be discovered was zebrafish Sef (ZSef), which consists of a putative transmembrane domain with a tyrosine phosphorylation site juxtaposed to the receptor (type I transmembrane receptor).6 Genomic sequence analysis revealed a 15–20% homology to the intracellular domain of the interleukin-17 receptor (IL17R),6,7 giving it the alternative name of interleukin-17 receptor D (IL17RD). Sef has since been identified in other vertebrates including chick (cSef), mouse (mSef) and human (hSef).8–11 The mechanism by which Sef regulates receptor tyrosine kinase (RTK) signaling remains contentious and is likely to be cell type specific. Tsang et al. in co-immunoprecipitation experiments have shown that ZSef interacts at the level of the FGF receptor.6 Kovalenko et al. have further shown that overexpression of mSef inhibited phosphorylation of FGFR1 and the adaptor protein FRS2.9 Sef interaction at the level of the receptor has also been shown in PC-12 rat medulla cells.12 Consistent with these findings, work in our unit in human prostate cancer has shown that hSef is likely to act at or above the level of Ras.13,14 Other groups however have proposed that hSef may function further downstream and potentially act as a spatial regulator of ERK.
What’s new?
The loss of negative signaling regulators may be implicated in prostate cancer metastasis, but the underlying mechanisms remain unclear. Here, by using a combination of xenograft, gene expression microarray, phosphokinase array and quantitative PCR techniques, the authors provide first evidence that the negative regulator hSe is plays a key role in regulating epithelial to mesenchymal transition (EMT) in prostate cancer, which in turn results in changes in the metastatic ability of tumor cells. The results support the notion that the expression levels of hSe and other negative signaling regulators may be key biomarkers for the identification of triggers for metastasis.

Material and Methods

Cell lines and xenograft experiments
PC3 cells were obtained from the American Type Culture Collection and the PC3M cell line (a metastatic derivative of PC3) was a gift from Professor H. Leung (Glasgow). PC3M cells were seeded into 90 mm tissue culture dishes and transfected with pCDNA3.1-Se-Myc (gift from Professor Z. Chang, Tsinghua University, China) or pcDNA3.1 (Invitrogen, UK) using Lipofectamine 2000 (Invitrogen, UK) before being placed under G418-sulphate selection for 14–20 days. Individual colonies were removed by trypsinisation and expanded. Cells were maintained in RPMI-1640 media (Sigma, UK) containing 10% foetal calf serum, termed full medium (FM). For xenograft studies two million PC3M-hSe or control cells (suspended in 50 μl of PBS and 50 μl of Matrigel solution) were injected subcutaneously into the lateral thoracic walls of NOD-SCID gamma mice (Charles River, UK; 10 each with PC3M-hSe and PC3M-EV cells). The tumor size at the injection site was measured weekly using calipers and was calculated using the formula:

\[ \text{Volume} = \frac{\pi}{6} \times a \times b \times c \]

where \( a, b, c \) are the orthogonal axis of the tumor. At autopsy (typically weeks 4–7), the primary tumor and lungs were harvested. In vivo experiments were reviewed and approved by the institutional animal welfare committee and performed according to the UKCCCR guidelines.

H&E and immunohistochemistry
Harvested tissue was fixed in 10% Neutral Buffered Formalin for 24 hrs and stored in 70% ETOH. H&E staining on the harvested lungs were performed and mounted on slides before image capture and storage. Immunohistochemistry against E-Cadherin and hSe was performed on the harvested xenograft tissue from the primary injection site using the BondMax Autostainer (Leica, UK). Briefly, the process involved the retrieval of antigen by heat retrieval (100°C) followed by incubation with anti E-Cadherin antibody (Novoceastra, UK) or anti hSe antibody (Sigma, UK) at a dilution of 1:25 or 1:500 at room temperature for 15 min, respectively. The samples were then incubated in a polymer secondary system (Leica) and developed with Diaminobenzidine using copper enhancement. All histopathology sections were scored by an experienced uropathologist (AW). The presence of metastasis was defined as >2 separate sites involved with tumor cells or a single area with >5 tumor cells.

Illumina microarray, EMT specific PCR array and real-time PCR
Total RNA was isolated from PC3M-hSe and PC3M-EV cells maintained in FM using the RNeasy Mini Kit (Qiagen, UK). Microarray experiments were performed using the HT12v4 beadchip (Illumina, UK). Gene ontology analysis on the resultant data was performed using Ingenuity Pathway Analysis (Qiagen, UK) and MetaCoreTM (Thomson Reuters, UK) software with all genes that were differentially expressed by ≥2 fold (up or downregulated) with \( p < 0.01 \) considered for analysis. Changes in expression of EMT related genes in PC3M-Se and PC3M-EV cells were investigated using the EMT specific RT2Profiler PCR Array (Qiagen, UK). In knockdown experiments PC3 cells were grown in FM for 24 hrs before being transfected with 33 nM of hSe siRNA or Scramble (Scr) control (Dharmacon, UK). ERK-MAPK inhibition experiments were performed using 30 μM of ERK inhibitor FR180204 (Sigma, UK) suspended in FM. Real-time PCR analysis were performed using primers obtained from the TaqMan® Gene Expression Assay.
In vivo, not only were there fewer metastatic deposits, but those large pulmonary deposits (lungs with nine out of ten xenografts showing evidence of lung metastasis (Fig. 1) with PC3M-hSef had definitive evidence of lung metastasis at autopsy. In total, only two out of ten xenografts injected with PC3M-hSef tumors grew at a much slower rate compared to PC3M-EV tumors (Fig. 1). The generated tumors exhibited significantly different dynamics with PC3M-Sef tumors growing at a much slower rate compared to PC3M-EV tumors (Fig. 1b). Harvested lung tissue was assessed for the presence of lung metastasis following autopsy. In total, only two out of ten xenografts injected with PC3M-hSef had definitive evidence of lung metastasis (Fig. 1c). In contrast, PC3M-EV readily metastasized to the lungs with nine out of ten xenografts showing evidence of large pulmonary deposits ($p < 0.01$). In PC3M-hSef bearing mice, not only were there fewer metastatic deposits, but those cells that did metastasize produced very small lesions in comparison to PC3M-EV (Fig. 1c). We further compared metastatic incidence matched for final tumor volume. In this analysis, 6/7 PC3M-EV xenografts (mean volume 1.25 cm$^3$) had lung micro-metastasis whilst only 1/6 PC3M-hSef (mean volume 1.27 cm$^3$) exhibited metastasis ($p = 0.03$). Collectively, these findings recapitulate the observations from our clinical studies of a role for hSef in influencing tumor metastasis.$^{13}$ This effect appears to be independent of primary tumor size.

### Western blotting and phosphokinase arrays

Cell lysates of hSef knockdown (or Sc control) PC3 cells were obtained at various time-points post stimulation with FM. Cells were lysed directly using SDS sample buffer containing 10% β-mercaptoethanol. Samples were denatured and separated using SDS–PAGE. Proteins were transferred onto a PVDF membrane and probed with primary antibodies against total or phosphorylated ERK1 and ERK2 (Cell Signaling Technologies, UK) followed by incubation in the appropriate horseradish peroxidase-conjugated anti-rabbit or anti-goat secondary antibodies (Amersham Life Science). Separate aliquots of these preparations were used in the R&D Systems (UK) Proteome Profiler Phosphokinase antibody array (PKA). Changes in phosphorylation were calculated against pre-stimulation samples using image quantification as per the manufacturer's instructions.

### Statistical analysis

Data was analysed using the student's t test for unpaired samples or Fisher's exact test for categorical data. $p < 0.05$ was considered statistically significant.

### Results

#### hSef inhibits tumor dissemination in an in vivo metastatic model

Previously, we have demonstrated that hSef expression is closely linked with tumor aggressiveness, with reduced expression being a feature of high-grade and metastatic clinical prostate cancer.$^{15}$ To corroborate this finding in vivo, a xenograft model was adapted from a previously reported protocol for prostate cancer lung metastasis using PC3M.$^{18}$ This cell line is known to have very low levels of endogenous hSef.$^{17}$ PC3M-EV and PC3M-Sef cells were used to produce lateral thoracic wall tumors in immune-deficient mice (Fig. 1a). The generated tumors exhibited significantly different dynamics with PC3M-Sef tumors growing at a much slower rate compared to PC3M-EV tumors (Fig. 1b). Harvested lung tissue was assessed for the presence of lung metastasis following autopsy. In total, only two out of ten xenografts injected with PC3M-hSef had definitive evidence of lung metastasis (Fig. 1c). In contrast, PC3M-EV readily metastasized to the lungs with nine out of ten xenografts showing evidence of large pulmonary deposits ($p < 0.01$). In PC3M-hSef bearing mice, not only were there fewer metastatic deposits, but those cells that did metastasize produced very small lesions in comparison to PC3M-EV (Fig. 1c). We further compared metastatic incidence matched for final tumor volume. In this analysis, 6/7 PC3M-EV xenografts (mean volume 1.25 cm$^3$) had lung micro-metastasis whilst only 1/6 PC3M-hSef (mean volume 1.27 cm$^3$) exhibited metastasis ($p = 0.03$). Collectively, these findings recapitulate the observations from our clinical studies of a role for hSef in influencing tumor metastasis.$^{13}$ This effect appears to be independent of primary tumor size.

#### hSef alters the expression of genes involved in EMT

Given our finding that hSef influences the metastatic ability of prostate cancer cells in vivo, we next sought to investigate the possible biological mechanisms by which this effect is permeated. For this, RNA extracted from PC3M-hSef and PC3M-EV cells following 24 hrs' stimulation with FM was interrogated for comparative gene expression using the Illumina HT12v4 beadchip. Gene network analysis revealed that the top network of genes most influenced by hSef were those involved in cellular movement, cancer, cellular growth and proliferation. Gene ontology analysis was refined further by enriching according to biological functions. This revealed that genes involved in cell adhesion, Extra Cellular Matrix remodeling and Epithelial to Mesenchymal Transition (EMT) appeared to be the most biologically altered by hSef.

We next pursued this observation by investigating the effect of hSef using an EMT gene specific PCR array. In this assay, hSef overexpression with FM stimulation resulted in a significant upregulation of E-Cadherin expression, a key adhesion molecule involved in EMT (Fig. 2a). In addition, a number of genes involved in upregulating EMT were concomitantly suppressed including SIP1, ZEB2, WNT5B, ITGA5, IGFBP4, STEAP1 and SNAI2 (Fig. 2a). The most markedly downregulated gene was Versican, a chondroitin sulphate proteoglycan known to increase cell migration, growth and metastasis.$^{19}$ We next tested the converse effect of hSef suppression on E-Cadherin using targeted siRNA in PC3 cells (known to express high levels of endogenous hSef$^{17}$ (Fig. 2b). qPCR analysis revealed that the knock-down of hSef in PC3 cells stimulated with FM resulted in a significant decrease (>80%) in the expression levels of E-Cadherin compared to non-targeting controls (Fig. 2c). Given the central role of hSef in attenuating the ERK-MAPK pathway, we next investigated whether hSef alters the expression of E-Cadherin through this pathway. To test this, the above experiment using PC3 control and hSef knockdown cells were repeated using an ERK inhibitor (FR182020). Our results revealed that ERK inhibition significantly reduced the downregulation of E-Cadherin expression that was seen as a result of hSef knockdown (Fig. 2d). Collectively, our results suggest that hSef influences E-Cadherin expression through modulation of the ERK-MAPK pathway.

To corroborate our observation that hSef influences E-Cadherin expression, histological sections obtained from the primary xenografts tumor sites were subjected to immunohistochemistry for E-Cadherin protein. On histological features
alone PC3M-hSef tumors appeared more densely packed compared to PC3M-EV (Fig. 2e). Concomitantly, PC3M-hSef expressed markedly higher levels of E-Cadherin compared to EV tumors (Fig. 2f). hSef expression simultaneously attenuates ERK, p38 and JNK pathways
The role of hSef in modulating the ERK-MAPK pathway is well described\textsuperscript{10,12–15} and our findings adds further weight to
the importance of this pathway in attenuating the expression of E-Cadherin, a key adhesion protein involved in the EMT process. It is currently not known however, whether hSef has any effect on other intracellular signaling pathways that are involved in mediating EMT such as p38 and JNK. To investigate this, hSef expression was suppressed using siRNA (or Scr controls) in PC3 cells and stimulated with FM before being tested in a phosphokinase array (PKA). We re-confirm the robustness of our model system by testing for ERK phosphorylation using western blot. As expected, hSef knockdown with FM stimulation resulted in a marked downregulation of E-Cadherin expression (>80%). Error bars represent the mean of three experiments done in triplicate. (e) H&E sections from the primary xenograft tumor injection sites. PC3M-EV cells were histologically more segregated in comparison with the hSef over-expressing PC3M cells. (f) Immunohistochemistry against hSef protein confirms high expression in PC3M-Sef but not in PC3M-EV tumors. E-Cadherin protein immunohistochemical expression was noted to be absent or very low in PC3M-EV tumors but increased in hSef over-expressing xenografts. Representative tumors are shown for both E and F. (*) $p < 0.05$, (**) $p < 0.001$.

Figure 2. hSef attenuates the expression of EMT genes. (a) EMT specific RT2 Profiler PCR array revealed that a number of genes involved in the process of EMT were de-regulated as a result of hSef over-expression. The gene most upregulated was E-Cadherin. One of three replicate experiments is shown. (b) siRNA against hSef was performed using PC3 prostate cancer cells with a >80% knock-down efficiency. (c) hSef knockdown with FM stimulation resulted in a marked downregulation of E-Cadherin expression (>80%). Error bars represent the mean of three experiments done in triplicate. (d) The experiment was repeated using an ERK-inhibitor (FR18020) which revealed that ERK signaling blockade significantly reduced the downregulation of E-Cadherin expression that was seen as a result of hSef knockdown. Error bars represent the mean of the experiment performed in triplicate. (e) H&E sections from the primary xenograft tumor injection sites. PC3M-EV cells were histologically more segregated in comparison with the hSef over-expressing PC3M cells. (f) Immunohistochemistry against hSef protein confirms high expression in PC3M-Sef but not in PC3M-EV tumors. E-Cadherin protein immunohistochemical expression was noted to be absent or very low in PC3M-EV tumors but increased in hSef over-expressing xenografts. Representative tumors are shown for both E and F. (*) $p < 0.05$, (**) $p < 0.001$.
resulted in a significant enhancement in both intensity and duration of ERK phosphorylation (Fig. 3a). This was matched in the PKA panel whereby hSef silencing resulted in a significant increase in the phosphorylation of ERK 1/2 compared to scramble transfected cells in response to FM stimulation particularly at the 15 and 60-min time-points (Fig. 3b). Across the rest of the panel, hSef silencing also resulted in enhanced phosphorylation of p38 (Fig. 3c) with a similar trend also seen for JNK (Fig. 3d). These results suggest that hSef simultaneously attenuates an array of intracellular signaling pathways, which are known to have a role in mediating EMT.

Discussion

Metastasis is a critical and devastating step in prostate cancer progression. The biological mechanisms that lead to prostate cancer metastasis remain poorly understood but a common final pathway is through activation of EMT. In addition to facilitating tumor dissemination, EMT has also been implicated in resistance to radiotherapy and chemotoxic agents. EMT is therefore an important target when considering novel agents in cancer therapy.

There is good evidence to implicate the loss of NSR in prostate cancer metastasis. hSef in particular, has been consistently shown to be downregulated in advanced and metastatic tumors. This downregulation were thought to be primarily due to changes in cellular growth and proliferation. In this study, we have demonstrated that altering hSef expression has a direct effect in modulating EMT. This is consistent with previous reports of a similar role for other NSR. RKIP1 overexpression, for instance, has been shown to suppress EMT in nasopharyngeal cancers. Members of the Sprouty family are known to repress TGFβ-induced EMT in the context of cataract development. Recent work from the same group has since shown a similar role for Spreds 1–3 and Sef in the same context. Furthermore, He et al. recently revealed that hSef plays a role in the negative regulation of EMT in a β-catenin dependent manner in breast cancer cells. These data corroborate and support our own findings in this study on the role of hSef in attenuating EMT in prostate cancer.

The exact mechanism by which hSef influences EMT in prostate cancer is currently unknown. Recently, hSef overexpression was found to prevent the downregulation of E-Cadherin in a normal breast epithelial cell line that was forced to undergo EMT in a TGFβ dependent manner. As TGFβ induced EMT occurs through canonical and noncanonical pathways, it is conceivable that hSef modulates EMT by attenuating the various MAPK signaling pathways as was seen in the present study. Specifically, in the context of E-Cadherin it appears that hSef influences its expression, at least in part, through the attenuation of the ERK-MAPK...
pathway. Further studies are required to elucidate the exact mechanism by which hSef influences EMT in prostate cancer.

In conclusion, we have demonstrated here first evidence that hSef has a key role in regulating EMT in prostate cancer, which in turn results in changes in the metastatic ability of tumor cells. This we have now demonstrated in vitro, in vivo and in clinical studies. These findings support the general notion that hSef and other NSR are important tumor suppressors and a loss of expression leads to a metastatic phenotype. Taken together, this argues strongly for the possibility that the expression levels of hSef and other NSR may be key biomarkers in identifying triggers for metastasis. In addition, while the notion of targeting loss of expression proteins is challenging in terms of therapy, overcoming this paradigm may provide a new class of therapeutic agents, which can inhibit tumor dissemination and therefore alter the natural history of the disease.

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