Knockdown of SPRY4 and SPRY4-IT1 inhibits cell growth and phosphorylation of Akt in human testicular germ cell tumours

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Testicular germ cell tumour (TGCT) is the most common cancer in young men in large parts of the world, but the aetiology is mainly unknown. Genome-wide association studies have so far identified about 50 susceptibility loci associated with TGCT, including SPRY4. SPRY4 has shown tumour suppressor activity in several cancer cells, such as lung and prostate, while it was found to act as an oncogene in ovarian cancer. An intronic region within the SPRY4 gene produces a long non-coding RNA, SPRY4-IT1, which has been reported to act as an oncogene in melanoma, breast cancer, and colorectal cancer, and as a tumour suppressor in lung cancer. The roles of SPRY4 and SPRY4-IT1 in TGCT development are yet unknown. We found higher expression levels of SPRY4, both mRNA and protein, and of SPRY4-IT1 in human TGCT than in normal adult testis. Small-interfering RNA (siRNA)-mediated transient knockdown of SPRY4 and SPRY4-IT1 in two TGCT cell lines 833 K and NT2-D1 resulted in decreased cell growth, migration, and invasion. Knockdown of SPRY4 and SPRY4-IT1 also led to a significant reduction in the phosphorylation of Akt. Our findings indicate that SPRY4 and SPRY4-IT1 may act as oncogenes in TGCTs via activation of the PI3K/Akt signalling pathway.

Testicular germ cell tumour (TGCT) is the most common malignancy in young men in large parts of the world. The incidence of TGCT has been increasing over the last decades and is highest in white Caucasian populations in industrialised countries and lowest in men of African ancestry1–3. The aetiology of TGCT is poorly understood. Both genetic and environmental factors are believed to contribute to the disease risk4,5, of which approximately 25% of TGCT susceptibility may be caused by genetic effects6,7. The precursor cell to TGCT, carcinoma in situ, resembles primordial germ cells, and there is epidemiological evidence indicating that TGCT originates in fetal life8,9. However, some studies suggest that environmental exposures in adolescence and adulthood are also associated with TGCT risk4,10.

Genome-wide association (GWA) studies of TGCT have so far identified about 50 susceptibility loci11–13, and SPRY4 is one of the genes, which shows strong and consistent association9,13–15. SPRY4 belongs to a family of four genes (SPRY1–4) encoding proteins which are well-known regulators of receptor tyrosine kinases (RTKs)16. The RTK-mediated MAPK/ERK and PI3K/Akt signalling pathways are involved in the homeostasis of cell growth and differentiation, and in cancer, activation of these pathways leads to increased cell proliferation, survival, invasion, and metastasis17. Altered expression of SPRY in cancer may cause aberrant regulation of MAPK/ERK and PI3/Akt signalling pathways18. Involvement of SPRY4 in the regulation of tumorigenesis has already been documented in several human cancer types19. In lung and prostate cancer, SPRY4 showed tumour suppressor activity20,21, whereas in ovarian cancer, knockdown of SPRY4 attenuated growth factor-induced cancer progression21. SPRY4 may also play a role in the regulation of cell growth and differentiation in TGCT pathogenesis. Moreover, SPRY4-IT1, a long non-coding RNA (lncRNA) produced within an intronic region of SPRY4, has been reported to promote cancer development in melanoma22, breast cancer23, and colorectal cancer24 while inhibiting lung cancer growth25. However, to our knowledge, no effect of SPRY4-IT1 on RTK-mediated signalling pathways has been reported.

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The roles of SPRY4 and SPRY4-IT1 in TGCT development are yet unknown. The lack of an appropriate laboratory animal model, as well as the difficulties of establishing primary cultures from human germ cells, makes studying human TGCT pathogenesis a challenge. In the present work, we used metastatic TGCT tissue-derived or laboratory animal model, as well as the difficulties of establishing primary cultures from human germ cells, make SPRY4 and SPRY4-IT1 to explore the roles of studying human TGCT pathogenesis a challenge. In the present work, we used metastatic TGCT tissue-derived or laboratory animal model, as well as the difficulties of establishing primary cultures from human germ cells, make SPRY4 and SPRY4-IT1 to explore the roles of SPRY4 and SPRY4-IT1 in metastatic TGCT tissue.

**Results**

**Expression of SPRY4 and SPRY4-IT1 in TGCT.** We examined the expression levels of SPRY4 and SPRY4-IT1 in 13 TGCTs and 11 normal testis samples. The TGCT samples comprised of yolk sac tumour (n = 3), embryonal carcinoma (n = 4), teratoma (n = 3), seminoma (n = 2), and choriocarcinoma (n = 1). RNA levels of SPRY4 and SPRY4-IT1 were significantly higher in all the TGCT samples than in normal testis samples (Fig. 1a). Furthermore, the SPRY4 and SPRY4-IT1 RNA levels in yolk sac tumour, embryonal carcinoma, and teratoma were considerably higher than those in choriocarcinoma and seminoma. There was also a notable difference between SPRY4 and SPRY4-IT1 expression patterns in TGCTs. SPRY4 expression levels were higher than those of SPRY4-IT1 in yolk sac tumour, embryonal carcinoma, and choriocarcinoma, whereas SPRY4-IT1 expression levels were higher than those of SPRY4 in teratoma. SPRY4 protein was also abundantly expressed in all TGCT samples, whereas no expression was detected in any of the normal testis samples (Fig. 1b). A profound difference of SPRY4 protein expression between moderately differentiated seminoma and undifferentiated seminoma was observed, with the highest level in undifferentiated seminoma.

**Knockdown of SPRY4 and SPRY4-IT1.** SPRY4 and SPRY4-IT1 were expressed in both NT2-D1 and 833 K cells. siRNA-mediated gene silencing resulted in an average of 75% reduction in SPRY4 and SPRY4-IT1 RNA expression levels in 833 K cells (Fig. 2a,c) and NT2-D1 cells (Fig. 2b,d). SPRY4 protein expression was also substantially decreased in both cell lines after knockdown (Fig. 2e), and densitometric analysis of the western blot shows that knockdown of SPRY4 protein was more efficient in NT2-D1 cells (Fig. 2f) than in 833 K cells (Fig. 2g).

**Effect of knockdown of SPRY4 and SPRY4-IT1 on cell growth, migration, and invasion.** Both SPRY4 and SPRY4-IT1 have been observed to alter cell growth, migration and invasion in prostate cancer and melanoma. To investigate the effects of SPRY4 and SPRY4-IT1 knockdown on TGCT cell growth, we performed cell counting and cell proliferation assay after the knockdown of SPRY4 and SPRY4-IT1 in the 833 K and NT2-D1 cells lines. Knockdown of both genes led to a significant decrease in viable cell number (Fig. 3a,b) and
cell proliferation (Fig. 3c,d) in a time-dependent manner relative to control. Knockdown of SPRY4 and SPRY4-IT1 also resulted in a significant reduction in cell migration (Fig. 4a,b) and invasion (Fig. 4c,d).

**Effect of knockdown of SPRY4 and SPRY4-IT1 on MAPK / ERK and PI3K / Akt signalling.** Since increased cell proliferation in malignancies happens through the aberrant activation of MAPK / ERK and PI3K / Akt signalling pathways\(^\text{17}\), we investigated whether knockdown of SPRY4 and SPRY4-IT1 altered the activation of...
MAPK / ERK and PI3/Akt pathways. Knockdown of SPRY4 and SPRY4-IT1 significantly inhibited the phosphorylation of Akt in both 833 K and NT2-D1 cells, while phosphorylation of ERK1/2 showed a weak but significant inhibition in NT2-D1 cells only after SPRY4 knockdown (Fig. 5a,b). We also examined the phosphorylation of Akt in the tissue and found phospho-Akt in all the TGCTs (Supplementary Fig. S5). A weak band was detected in one of the normal samples of comparable intensity with the one in moderately differentiated seminoma.

Discussion
In our study, the finding of high expression of SPRY4 and SPRY4-IT1 in TGCTs compared with normal adult testis strengthens the hypothesis of a role of the SPRY4 gene in TGCT development. The distinct difference in expression between TGCT and normal tissue was also observed for the SPRY4 protein. Particularly, no detection of SPRY4 protein in any normal testis sample, and the profound difference of SPRY4 protein expression between moderately differentiated seminoma sample and undifferentiated seminoma sample indicate that SPRY4 may act as an oncogene in TGCT pathogenesis. We found lower RNA expression levels of SPRY4 and SPRY4-IT1 in seminoma than in other TGCT subtypes but had no information about the status of differentiation of these two seminoma samples. The only report of higher expression of SPRY4 and SPRY4-IT1 in malignant tissue than in the normal is from human melanoma tissue relative to melanocytes. Genetic knockdown of SPRY4 and SPRY4-IT1 in 833 K and NT2-D1 cells resulted in a decrease in cell survival, proliferation, migration, and invasion. This is in accordance with a study in human ovarian cancer cells where suppression of SPRY4 attenuated growth factor-induced cell migration and invasion. Furthermore, suppression of SPRY4-IT1 in human melanoma cells inhibited cell growth, migration, and invasion, indicating oncogenic activity. However, an opposite role of SPRY4 and SPRY4-IT1 has been reported in other types of cancer. For example, SPRY4 showed tumour suppressor activity in lung, prostate, and breast cancer, and SPRY4-IT1 showed tumour suppressor activity in lung cancer.
Figure 5. Effects of SPRY4 and SPRY4-IT1 knockdown on the activation of PI3/Akt and MAPK/ERK1/2 pathway. Western blot was performed to investigate the phosphorylation of Akt and ERK1/2 after knockdown of SPRY4 and SPRY4-IT1. After knockdown of SPRY4 and SPRY4-IT1, Akt phosphorylation was significantly inhibited in 833 K (a) and NT2-D1 (b) cells, particularly, inhibition of Akt phosphorylation was quite substantial in NT2-D1 cells by both SPRY4 and SPRY4-IT1 knockdown. However, phosphorylation of ERK1/2 only showed a weak but significant inhibition in NT2-D1 cells after the SPRY4 knockdown. Akt and ERK1/2 were used as endogenous controls, and α-tubulin was used as a loading control. The bar graphs show the corresponding densitometric analyses of the western blots where the ratio of p-Akt/Akt, and the ratio of p-ERK1/2/ERK1/2 were calculated after normalising with α-tubulin. Samples were loaded as independent duplicates. The cropped blots are used in the figure, and full-length blots are presented in Supplementary Figs S3, 4. The experiments were repeated at least three times, and a representative experiment is shown. *t-test: Control vs siRNA, mean ± SD, statistical significance p < 0.05.

In malignancies, the role of SPRY gene family on RTK-mediated MAPK / ERK and PI3K / Akt signalling seems to be cell-specific and context-dependent. In our study, silencing of SPRY4 and SPRY4-IT1 in both the TGCT cell lines displayed a potent inhibition of Akt phosphorylation. Phospho-Akt was detected in all the TGCTs, but only in one normal testis sample. Furthermore, the level was low in both the normal sample and the moderately differentiated seminoma. These findings support that activation of PI3K / Akt signalling plays a role in TGCT development, as also reported in another study. Furthermore, SPRY4 knockdown in NT2-D1 cells resulted in a weak but significant inhibition of ERK1/2 phosphorylation. The degree of SPRY4 knockdown attained in the 833 K cells might not be sufficient to observe an inhibitory effect on phosphorylation of ERK1/2. To our knowledge, this is the first study to show an inhibitory effect of SPRY4 and SPRY4-IT1 knockdown on RTK signalling in any cancer cell type. In contrast to our findings, suppressing SPRY4 in human breast carcinoma cells resulted in a substantial increase in phosphorylation of Akt and a mild increase in phosphorylation of ERK1/2. Interestingly, the regulatory function of SPRY4 on RTK signalling has been shown to be ligand-specific. In human embryonic kidney fibroblasts and mouse embryonic fibroblasts, SPRY4 decreased the FGF-induced activation of ERK1/2 but increased the EGF-induced ERK1/2 activation. As far as we know, the effect of SPRY4-IT1 on RTK signalling in any other cell type has not been reported.

Protein-coding genes and their associated intronic lncRNAs usually display positively correlated expression profiles. Khaitan et al. reported a positive correlation between the expressions of SPRY4 and SPRY4-IT1 across different normal tissues and melanoma patient tissues. In normal tissue, SPRY4-IT1 showed higher expression than SPRY4 and also larger variation. In the melanoma samples, however, the expression level of SPRY4 was higher than that of SPRY4-IT1 in some samples and vice versa in others. In line with this, we found higher expression of SPRY4 than that of SPRY4-IT1 in three of the TGCT subtypes, whereas, in teratoma, the expression of SPRY4-IT1 was higher than that of SPRY4. The higher levels of the host gene SPRY4 and its intronic lncRNA SPRY4-IT1 with variable expression patterns in different TGCT subtypes suggest they are not simply oncogenes but rather have some independent regulatory mechanisms. In a follow-up study by Khaitan’s group, Mazar et al. showed that genetic knockdown of neither SPRY4 nor SPRY4-IT1 altered the expression of the other and that SPRY4-IT1 displayed stronger response to growth factors compared to SPRY4. Furthermore, they observed that SPRY4 and SPRY4-IT1 transcript decay was independently regulated. They also found that human melanoma cell invasion was 50% reduced by SPRY4-IT1 knockdown but unaffected by the SPRY4 knockdown and that SPRY4-IT1 silencing induced apoptosis more effectively than did SPRY4 knockdown. These findings indicate both transcriptional and functional independence of the host gene SPRY4 and its lncRNA SPRY4-IT1. However, in our study, we showed similar effects of SPRY4 and SPRY4-IT1.
A challenge in performing expression studies in TGCTs is the limited availability of healthy adult testis tissue to be used as a control. Researchers often use tissue adjacent to tumour tissue as a normal control, which may have cancer-associated genetic characteristics and raises questions. The use of normal testis samples is a strength in our study. Results from in vitro studies may not be the representative of the situation in vivo. However, there is no appropriate in vivo model for human TGCT, and no animal model has so far been able to form the precursor carcinoma in situ cells observed in human. The cell lines NT2-D1 and 833 K used in our study were derived from human metastatic TGCT tissues of lung and abdomen, respectively. Both TGCT cell lines exhibit characteristics similar to EC cells, which represent the pluripotent stem cells of teratocarcinomas. NT2-D1 cells also have common characteristics with teratoma and 833 K cells with teratoma, yolk sac tumour, and seminoma. EC cell lines have received the most attention as an experimental model for functional studies of TGCT in lack of suitable animal models.

Our results suggest that knockdown of both SPRY4 and SPRY4-IT1 inhibit TGCT growth by inhibiting the activation of PI3K / Akt pathway (Fig. 6), thus, acting as oncogenes. Further mechanistic studies of SPRY4 and SPRY4-IT1 are needed to get more knowledge about TGCT pathogenesis. Studies of the interaction with proteins encoded by other susceptibility genes, such as KITLG, BAK1 and DMRT1, may also advance the understanding of the mechanisms behind TGCT development.

**Methods**

**Tissue.** For the expression analysis, 13 TGCT samples of different patients were provided by Dr Rolf I. Skotheim (Genome Biology Group, Oslo University Hospital), consisting of yolk sac tumour (n = 3), embryonal carcinoma (n = 4), teratoma (n = 3), seminoma (n = 2), and choriocarcinoma (1) subtypes. Normal testicular tissue samples were collected from 11 adult organ transplant donors with no known history of cancer. For immunoblotting, a series of protein lysates made from TGCT tissues was bought from Protein Biotechnologies (CA, USA). The study has been approved by the Regional Committee for Medical and Health Research Ethics, Norway (2016 / 2006, REC South-East), and all experiments were performed in accordance with approved guidelines and regulations. Donors of the TGCT samples provided informed written consent, and for the normal testis samples in connection with organ transplantation, consent was obtained according to the Norwegian legislation relating to transplantation, hospital autopsies and the donation of bodies.

**Cell culture.** Two TGCT cell lines, NT2-D1 and 833 K were kindly provided by Dr Birgitte Lindeman (Norwegian Institute of Public Health, Oslo). 833 K and NT2-D1 were cultured in RPMI-1640 and DMEM medium, respectively, supplemented with 10% foetal bovine serum at 37 °C in a humidified 5% CO₂ incubator. The morphology of the cells was regularly checked, and stocks of cell lines were passaged not more than ten times for use in experiments.

**Knockdown experiments.** To knockdown the expression of SPRY4 and SPRY4-IT1 in 833 K and NT2-D1 cells, small-interfering RNA (siRNA) based gene silencing technology was applied. siRNA transfection protocol was adopted from Felfly et al. with slight modification. Cells were seeded out in a 6-well plate and grown overnight. Lipofectamine RNAiMAX (Invitrogen, CA, USA) transfection mix was prepared with siRNA sets (Supplementary Table S1) and applied to 833 K and NT2-D1 cells to knockdown the expression of SPRY4 and SPRY4-IT1. 100% transfection efficiency was confirmed by using a plasmid encoding green fluorescent protein. The expression of this protein in transfected cells was detected by fluorescence microscopy (data not shown). After 48 hours of transfection, cells were harvested and stored at −70 °C until further use. Knockdown was verified using qPCR and western blot.

**Quantitative PCR (qPCR).** RNA from cell lines and tissue samples were extracted using RNeasy (Qiagen, CA, USA), and 100 ng of RNA was converted to cDNA using TaqMan Reverse Transcription Reagents Kit.
(Applied Biosystems, CA, USA). qPCR was performed using 0.5 ng of cDNA and TaqMan Pre-Developed Assay Reagents (Applied Biosystems, CA, USA) under recommended conditions on a Mx3005P instrument (Agilent Technologies, Santa Clara, USA). All samples were run in triplicates, and the relative expression was calculated using the equation \( \Delta \Delta C_t \). CT values > 35 were regarded as negative. RPS29 has been shown to be stably expressed in adult human testis and germ cell neoplasms\(^{[46]}\) and was used as a reference gene in our study. The primers used are listed in Supplementary Table S2.

**Western blot**. Proteins were isolated after transfection with siRNAs using RIPA buffer (SIGMA-ALDRICH) containing 150 mM NaCl, 1.0% IGEPA\(\text{L}^\circ\) CA-630, 0.5% sodium deoxycholate, 0.1% SDS, 50 mM Tris, pH 8.0, phosphatase inhibitors and protease inhibitors. The protein concentration was measured using protein assay dye reagent concentrate (BioRad), and 30 µg protein was loaded onto 10% Mini-PROTEAN\(\text{Tm} \) TGX Precast Gels (Bio-rad), unless otherwise specified (Supplement Fig. S5). After SDS-PAGE, the proteins were blotted onto a PVDF membrane, and the membrane was blocked in TBST with 5% skim milk before incubating with primary antibody overnight at 4 °C. An HRP conjugated secondary antibody was used, and the proteins were detected using the ImageLab machine (BioRad). Optical density (OD) of the protein bands was determined by using Image Studio Lite. Primary antibodies used in this study were: Anti-β\(\text{catenin} \) (Cell Signaling, 1:2000), Anti-Akt (Cell Signaling, 1:1000), Anti-phospho-Akt (ser473) (Cell Signaling, 1:2000), Anti-ERK1/2 (Cell Signaling, 1:1000), Anti-phospho-ERK (ser378) (Cell Signaling, 1:2000), Anti-Akt (Cell Signaling, 1:1000), Anti-SPRY4 (Abcam, 1:1000). HRP-conjugated anti-rabbit (Cell Signaling, 1:2500) was used as secondary antibody.

**Cell counting**. Cells (120000) were seeded out in 6-well plates and grown overnight. After 48 hours of transfection with siRNAs, viable cells were counted during a period of five days by use of a haemocytometer. The cells were stained with trypan blue before counting to exclude dead cells.

**Cell proliferation**. The proliferative capacity of the cells was examined by XTT assay (Roche). After 48 hours of transfection with siRNAs, cells were seeded out in a 96-well plate and cultured at a density of 3 \( \times \) 10\(^4\) cells/ well. After a period of five days’ incubation, the cells were treated with 50 ul of XTT solution. The absorbance was measured at 450 nm with a microplate reader after 24 hours of incubation.

**Cell migration and invasion**. For the cell migration and invasion assays, cells were seeded out and cultured overnight, followed by siRNAs transfection and serum-deprivation for 24 hours. The cells were then harvested and assayed in a 96-well Boyden Chamber (R&D Systems) for migration and invasion according to manufacturer’s protocol.

**Statistical analysis**. The results were analysed by t-test using the PRISM software. Significant differences were defined by P-values < 0.05.

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

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**Author Contributions**

T.B.H. and K.F. are responsible for the conception of the study, and T.H.B., K.F. and M.K.D. are responsible for the experimental design. M.K.D., H.F.E. and O.P.H. performed the experiments, and all the authors interpreted data. M.K.D. was responsible for drafting the manuscript, and all authors critically revised the manuscript and approved of the final version to be published.

**Additional Information**

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