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Effects of TGF-β signaling in clear cell renal cell carcinoma cells

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ABSTRACT

Clear cell renal cell carcinoma (ccRCC) is by far the most common type of kidney cancer and is characterized by loss of the tumor suppressor gene *von Hippel–Lindau* (*VHL*). ccRCC patients with metastatic disease has poor prognosis and today's therapy is insufficient. The cytokine Transforming Growth Factor-β (TGF-β) has been extensively studied in tumor biology and is believed to serve a variety of functions in tumor progression. We have previously shown that inhibition of NOTCH signaling causes a reduced migratory and invasive capacity of ccRCC cells, at least partly by a cross-talk with the TGF-β pathway. In the present study we aimed to further clarify the role of TGF-β signaling in ccRCC. We investigated the effects of TGF-β pathway modulation and showed that TGF-β inhibition attenuates the invasive capacity of ccRCC cells. By performing expression profiling we obtained a gene signature of the TGF-β induced response in ccRCC cells. The expression analyses revealed an extensive overlap between the TGF-β response and genes regulated by the hypoxia inducible factor (HIF). The link between the hypoxic and the TGF-β pathways was further corroborated by functional experiments, which demonstrated that TGF-β pathway activity was attenuated upon reintroduction of functional VHL in ccRCC.

1. Introduction

Renal cell carcinomas (RCCs) comprise a histologically diverse group of solid tumors, arising from different parts of the kidney [1,2]. The vast majority of RCCs are clear cell renal cell carcinomas (ccRCCs), characterized by loss of function of the tumor suppressor gene *von Hippel Lindau* (*VHL*). Defects in the *VHL* gene are the most common cause of familial ccRCC, and more than 80% of patients with sporadic ccRCC have an inactive *VHL* gene [2,3]. An important function of the VHL protein is to target hypoxia-inducible factors (HIF-1α and HIF-2α) for degradation [4]. In the case of hypoxia, or loss of VHL function, HIF is stabilized which leads to the characteristic hypoxia response, including activation of genes involved in angiogenesis, glucose uptake, and metabolism [5,6]. In case of familial ccRCC, loss of *VHL* is manifested in the formation of renal cysts from which ccRCCs are thought to arise [7–9]. Nevertheless, it has been proposed that malfunction of VHL is accompanied by additional oncogenic events required for the formation and progression of the disease [10]. Metastasized disease represents formidable clinical challenge since disseminated tumors are refractory to conventional therapies, such as chemotherapy and radiation.

The transforming growth factor-β (TGF-β) pathway is involved in a plethora of cellular processes such as proliferation, migration, and apoptosis, as well as embryonal development, tissue repair, and inflammation. The TGF-β signaling cascade is initiated when TGF-β ligand binds to TGF-β Receptor type II (TβRII) which in turn recruits and phosphorylates TGF-β Receptor type I (TβRI). The phosphorylated TβRI then phosphorylates Smad2 and Smad3, which form a complex with Smad4. The Smad complex is transported into the nucleus where it, together with other transcription factors and co-factors, regulate transcription of target genes [11]. The TGF-β signaling pathway has contradictory roles in tumorigenesis. At early stages TGF-β acts as a tumor suppressor through activation of cell cycle inhibitors and activation of apoptosis. However, as the tumor progress the cytostatic effect is evaded and TGF-β instead conveys tumor-promoting signaling. For example TGF-β has been shown to facilitate immunosuppressive functions, enhance the invasiveness and angiogenesis, and initiate the process of epithelial to mesenchymal transition (EMT). The pro-oncogenic activities of TGF-β are exerted both on the microenvironment and tumor cells, making the tumor more aggressive and prone for dissemination [12,13]. In ccRCC, elevated expression of TGF-β1 is correlated to poor prognosis and the loss of VHL has been associated with modulations of the TGF-β1 expression [14–17]. We have shown that primary ccRCC cells treated with TGF-β1 acquires a mesenchymal phenotype with characteristics of EMT, thus corroborating the oncogenic influence of TGF-β on ccRCC [18]. Yet, a detailed characterization of the functional role and downstream effects of TGF-β in ccRCC is still lacking. In this study we aim to clarify this matter by analyzing changes in global gene expression as well as functional effects upon modulation of the TGF-β pathway in ccRCC cells.

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2. Materials and methods

2.1. Cell culture

The ccRCC cell lines SKRC-7, The SKRC-10, SKRC-17 were maintained in RPMI 1640 (Invitrogen™) containing 10% fetal calf serum (FCS, Invitrogen™) and 1% penicillin and streptomycin (PEST, Invitrogen™). 786-O cells (ATCC, Rockville, MD, USA) were maintained in DMEM High Glucose (GIBCO®, Invitrogen™) supplemented with 10% FCS and 1% PEST. The 786-O sub-clones pRC3, stably transfected with pRC/CMV, and WT7, stably transfected with CMV-HA-VHL and maintained as above with addition of 1 mg/ml G418 (GIBCO®, Invitrogen™). ccRCC cell line SKRC-52 was maintained in RPMI 1640 (Invitrogen™) containing 10% FCS and 1% PEST.

2.2. TGF-β1 and SB431452 treatment

Cells were treated with 0.25 ng/mL of human recombinant TGF-β1 (PeproTech) dissolved in 2 mg/mL BSA, or with 2 μM TβRI inhibitor, SB431542 (Sigma Aldrich) dissolved in DMSO. DMSO and/or 2 mg/mL BSA was used as vehicle control.

2.3. Proliferation WST-1

Cells were seeded in 96-well plates in 10% FCS and incubated over night (ON). Medium was changed to FCS-free medium and supplemented with 2 μM SB431542 or DMSO. After 24 h WST-1 dye reagent (Roche) was added to the cells and incubated for 4 h at 37 °C. The samples were analyzed in an ELISA plate reader (*Synergy 2*, BioTek) at 480 nm.

2.4. Western blot

Cells were maintained in FCS-free medium for 24 h followed by treatment with SB431542, TGF-β1, or vehicle control for 4 h before harvest. The cells were lysed, separated on a SDS–PAGE gel, and blotted onto a PVDF membrane (GE Healthcare), followed by incubation with anti-phosphorylated Smad2 antibody (Cell Signaling Technology), and re-blotted for anti-Smad2 (Cell Signaling Technology) and anti-Actin (MP Biomedicals, LLC). Mouse or rabbit ECL™ horseradish-peroxidase linked secondary antibodies (GE Healthcare). Proteins were detected by Luminol Reagent (Santa Cruz Biotechnology).

2.5. Elisa

Cells were seeded in 10% FCS medium and incubated ON. Cells were then maintained in FCS-free medium for 48 h. ELISA was performed using the Human TGF-β1 immuno assay (R&D Systems) according to manufacturer's description. An ELISA reader was used to analyze the absorbance at 450 nm.

2.6. Luciferase reporter assay

Cells were seeded in 6-well plates 24 h prior transfection with the $(CAGA)_{12}$-Luc, a vector containing 12 CAGA Smad binding sites using Liopfectamine 2000 (Invitrogen™) transfection reagent for 5 h in OptiMEM I Reduced Serum Medium (Invitrogen™). phRL-TK renilla expression vector (Promega) was used as a control for transfection efficiency. Following transfection, cells were treated with TGF-β1 or SB431542 for 4 h, then lysed and assayed for luciferase and renilla activity using the Dual Luciferase Reporter Assay System (Promega) and analyzed in an ELISA plate reader.

2.7. Invasion assays

Cells were pre-treated with SB431542 or DMSO control in FCS-free medium for 24 h before initiation of the assay. Cells were thereafter seeded in FCS-free medium supplemented with SB431542 or DMSO in Boyden Chambers with 8 μm pore size (Corning), coated with 50 μL of 12.5% Growth Factor Reduced BD Matrigel TM Matrix (BD Biosciences) diluted in FCS-free media. Cells were allowed to invade through the matrigel towards the lower compartment containing 10% FCS for 16 h. Invading cells were fixed on the membrane using 4% paraformaldehyde (Sigma Aldrich). The membranes were mounted onto a glass slide, stained with DAPI (Sigma Aldrich), and number of cells was determined using Image ProPlus software (Media Cybernetics). Three representative fields were counted for each membrane, and each treatment condition was assayed in triplicate and repeated three times.

2.8. Gene expression profiling

SKRC-10, SKRC-7 and SKRC-17 cells were seeded in 1% FCS ON followed by treatment with TGF-β, SB431542, or vehicle control for 8 h. Cells were harvested using Trizol reagent (Invitrogen) and RNA was isolated using the RNeasy micro kit (QIagen). Each experiment was carried out in quadruple, and for each sample, 250 ng of total RNA was hybridized to Illumina humanHT-12 v4.0 Expression BeadChips (Illumina Inc) at the SCIBLU Genomics Centre at Lund University. Data pre-processing and normalization was performed as described previously [19]. Differential gene expression between TGF-β and SB431542 treated cells was determined using the limma R package [20]. *P* values were adjusted using the Benjamini and Hochberg method. Gene Set Enrichment Analysis (GSEA) was performed as described previously [21] using the c2 curated gene sets as supplied by the Molecular Signatures Database (www.broad.mit.edu/gsea/msigdb), as well as to additional published TGF-β and HIF related gene sets [22–25]. The list of 157 TGF-β responsive genes was used to calculate a TGF-β specific pathway score for each sample in a ccRCC data set comprising 176 tumors [26], as described previously [17]. Patients were divided into two groups based on their relative TGF-β pathway activity score (above or below median) and Kaplan–Meier curves were compared using the log-rank test. Follow-up time was limited to ten years.

3. Results

3.1. TGF-β activity in CCRCC cells

We compared the secretion of TGF-β1 in a series of ccRCC cell lines, including SKRC-7, SKRC-10, SKRC-17, and SKRC-52. All investigated cell lines secreted TGF-β1 within the range previously reported for primary ccRCC cell cultures (Fig. 1A) [27]. To dissect the functionality of the TGF-β pathway in these cells we performed luciferase reporter analyses using a Smad regulated reporter gene $(CAGA)_{12}$-*Luc*). Both SKRC-7 and SKRC-52 responded with a significant induction of reporter gene activity upon treatment with TGF-β1, while SKRC-17 remained refractory to this treatment (Fig. 1B). The TβRI inhibitor SB431542 reduced baseline reporter gene activity in both SKRC-17 and SKRC-52 cells while no effect could be detected in SKRC-7 cells. In order to validate these observations we performed Western blot analyses of total and phosphorylated levels of Smad2 (P-Smad2). While all three cell lines expressed Smad2, only SKRC-7 and SKRC-52 responded with increased P-Smad2 upon TGF-β stimulation. Baseline levels of P-Smad2 were however decreased in all three cell lines upon treatment with SB431542 (Fig. 1C).

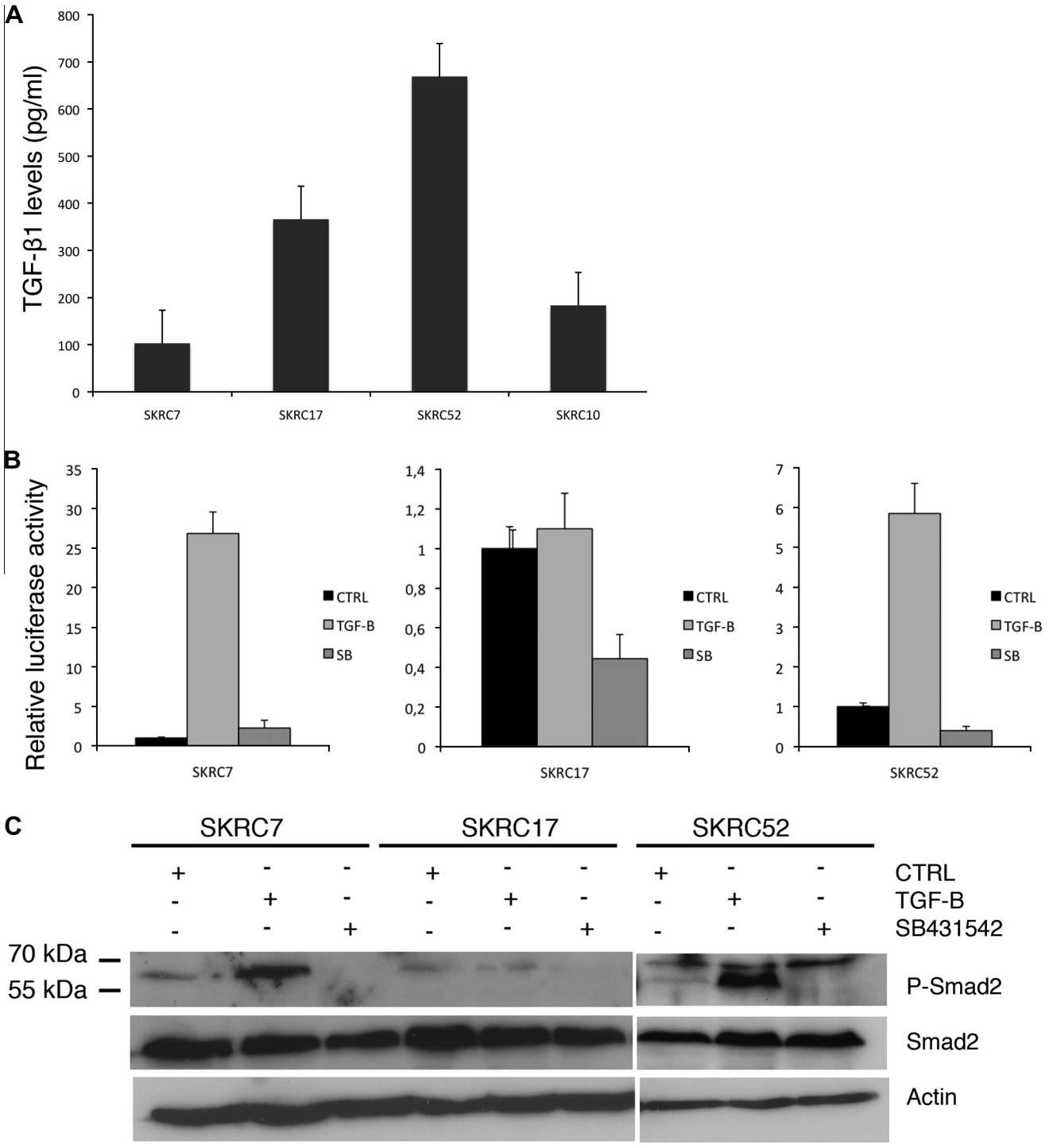

Fig. 1. (A) Quantitative determination of activated TGF-β1 concentrations by ELISA. (B) Relative luciferase activity of $(CAGA)_{12}$-Luc reporter in cells treated with TGF-β1, SB431542 or vehicle. Data was normalized to control treated cells. (C) Cells were treated with either TGF-β1, SB431542 or vehicle control and phosphorylated Smad2 was analyzed by Western blot. The membrane was re-probed with anti-Smad2 and anti-Actin antibodies. Data represent mean + SEM of three separate experiments.

3.2. Inhibition of TGF-βRI does not affect cell proliferation but reduce the invasive capacity of ccRCC cells

In light of the endogenous expression of TGF-β1 in ccRCC cells we next analyzed whether inhibition of this pathway affects proliferation. No significant impact on proliferation could, however, be detected when ccRCC cells were treated with the TβRI inhibitor SB431542 for 24 h. This result strengthens our previous observation that ccRCC cells evade the cytostatic effect of TGF-β signaling and that the tumor-promoting effect associated with TGF-β signaling is not related to proliferative changes (Fig. 2A). It has been established that induction of TGF-β signaling can induce the process of epithelial-to-mesenchymal transition (EMT), which is strongly associated with the metastatic progression of tumors [18,28,29]. We therefore assessed the invasive capacity of ccRCC cells using matrigel coated Boyden chambers upon inhibition of the TβRI. The number of invaded SKRC-7 and SKRC-10 cells was significantly decreased upon TβRI inhibition (Fig. 2B and C). These results imply that the invasive capacity of ccRCC cells, at least in part, is dependent on TGF-β signaling.

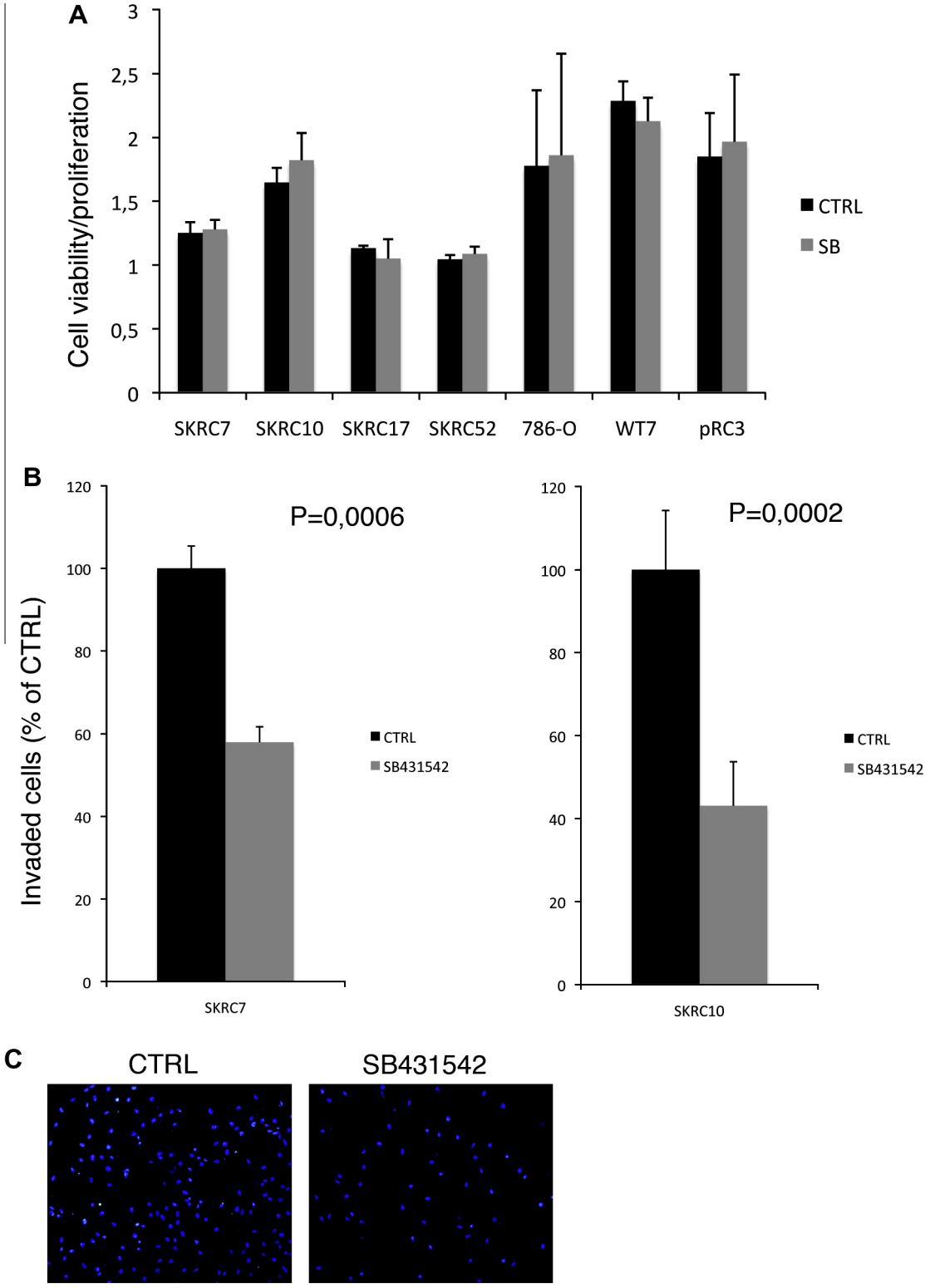

Fig. 2. (A) Cells were treated with TβR1 inhibitor SB431542 for 24 h before proliferation was analyzed by WST-1 assay. Data represent mean + SEM of three separate experiments. (B) Cell invasion was assessed by matrigel coated Boyden chamber assay of SKRC-7 and SKRC-10 cells. The cells were pre-treated with SB431542 or vehicle control. Data was normalized to control treated cells and represent mean + SEM of three experiments. (C) Representative images of invaded, SB431542 treated and control treated, SKRC-7 cells stained with DAPI.

3.3. TGF-β gene signatures in CCRCC

To broaden our understanding of the effects of TGF-β signaling of ccRCC we performed microarray based global gene expression profiling, comparing SKRC-10 and SKRC-7 cells treated with TGF-β1 to cells treated with SB431542. Through this approach we identified 157 genes that significantly differed in expression (Supplemental Table 1). In order to assess the generalizability of this ccRCC-specific TGF-β response, we first performed a gene set enrichment analysis (GSEA) comparing our data with previously

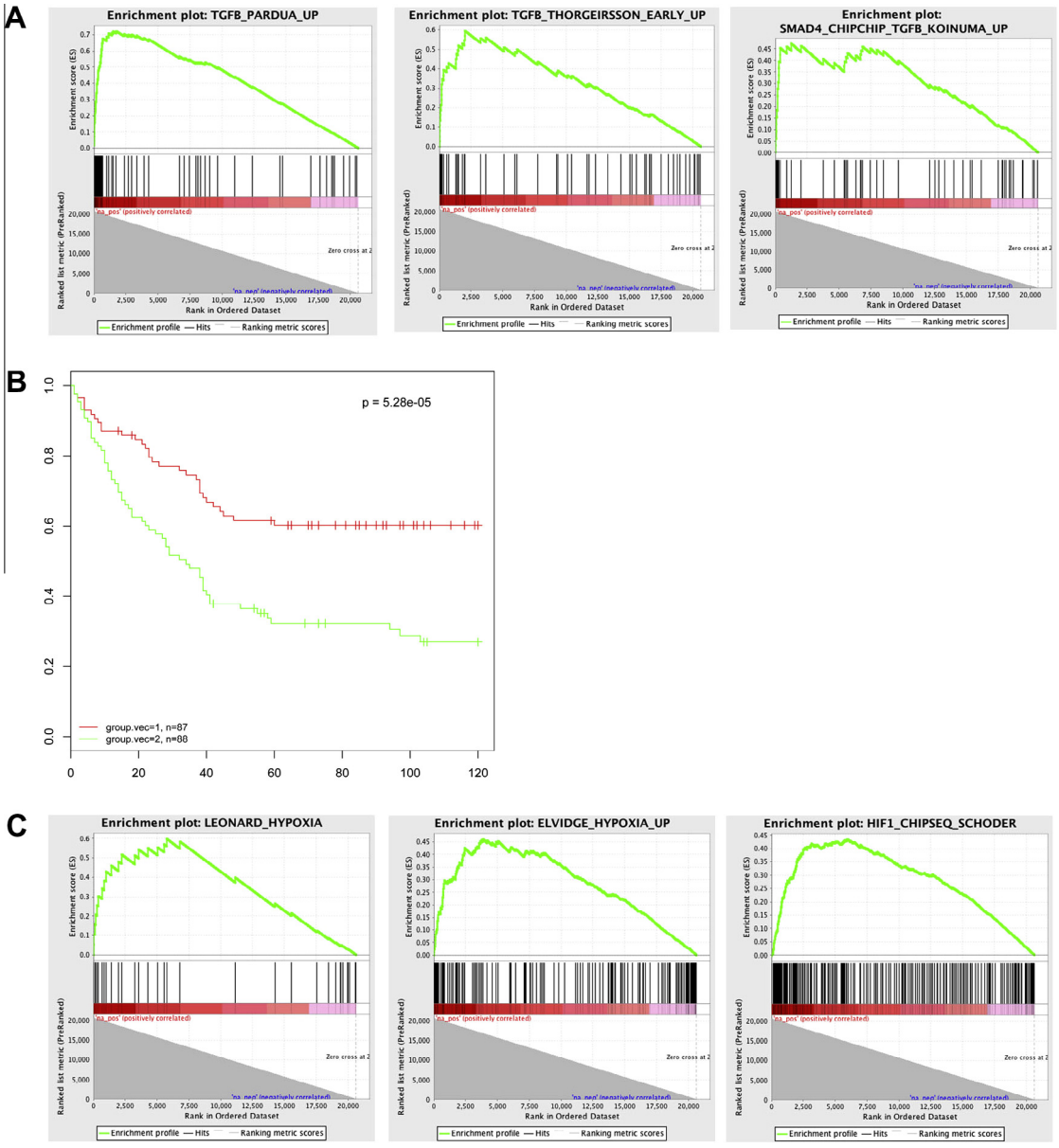

Fig. 3. Total RNA from SKRC-7 and SKRC-10 cells treated with TGF-β1 or SB431542, was used in oligomer microarry. (A) GSEA of the obtained TGF-β gene set compared with gene signatures of TGF-β treatment in other cell systems. (B) High ccRCC-specific gene signature correlates with adverse prognosis in ccRCC patients. Kaplan Meier curves comparing disease-specific survival in a cohort of 176 ccRCC patients divided in two groups based on a TGF-β signature score. (C) GSEA analyses from the MiSig database on hypoxia related signatures and a *bona fide* HIF-target gene signature derived from ChIP-seq analyses.

published gene signatures representing transcriptional responses to TGF-β treatment. This analysis revealed extensive similarities between the TGF-β response in ccRCC and other cell systems (Fig. 3A) [22–24]. We next used the 157-gene signature to calculate a TGF-β pathway activity score for each sample in a previously published gene expression data set comprising 176 ccRCCs with full clinical follow-up [26]. Significant correlation between high TGF-β pathway activity and adverse outcome was observed (Fig. 3B). This result corroborates our previous finding that a gene signature, comprising genes regulated by both NOTCH and TGF-β, was associated with poor prognosis. Together these results emphasize the association between elevated TGF-β activity and adverse prognosis of ccRCC.

It has been suggested that a cross talk exists between hypoxia-stimulated signaling (VHL/HIF) and TGF-β/Smad signaling [25]. However, the synergistic interactions between these two pathways remain poorly understood. Importantly, when performing unsupervised GSEA analyses of the 157-gene set using the MiSig database we noted significant enrichment for several hypoxia related signatures (Fig. 3C). Significant enrichment was also observed when applying a *bona fide* HIF-target gene signature derived from ChIP-seq analyses [25] (Fig. 3C), thus implying a commonality between the cellular responses to HIF and TGF-β.

3.4. Loss of VHL exacerbate the TGF-β1 signaling activity in ccRCC cells

The extensive overlap between HIF-regulated genes and genes modulated by TGF-β, prompted us to assess possible functional links between loss of *VHL* and elevated TGF-β pathway activity. We therefore analyzed the level of TGF-β1 secretion in the matched control (pRC3) and *VHL* reconstituted (WT7) 786-O ccRCC cell line derivatives. The WT7 cells secreted significantly lower levels of TGF-β1 compared to the control pRC3 cells (Fig. 4A) suggesting that the *VHL* status affects the secretion of TGF-β1, well in line

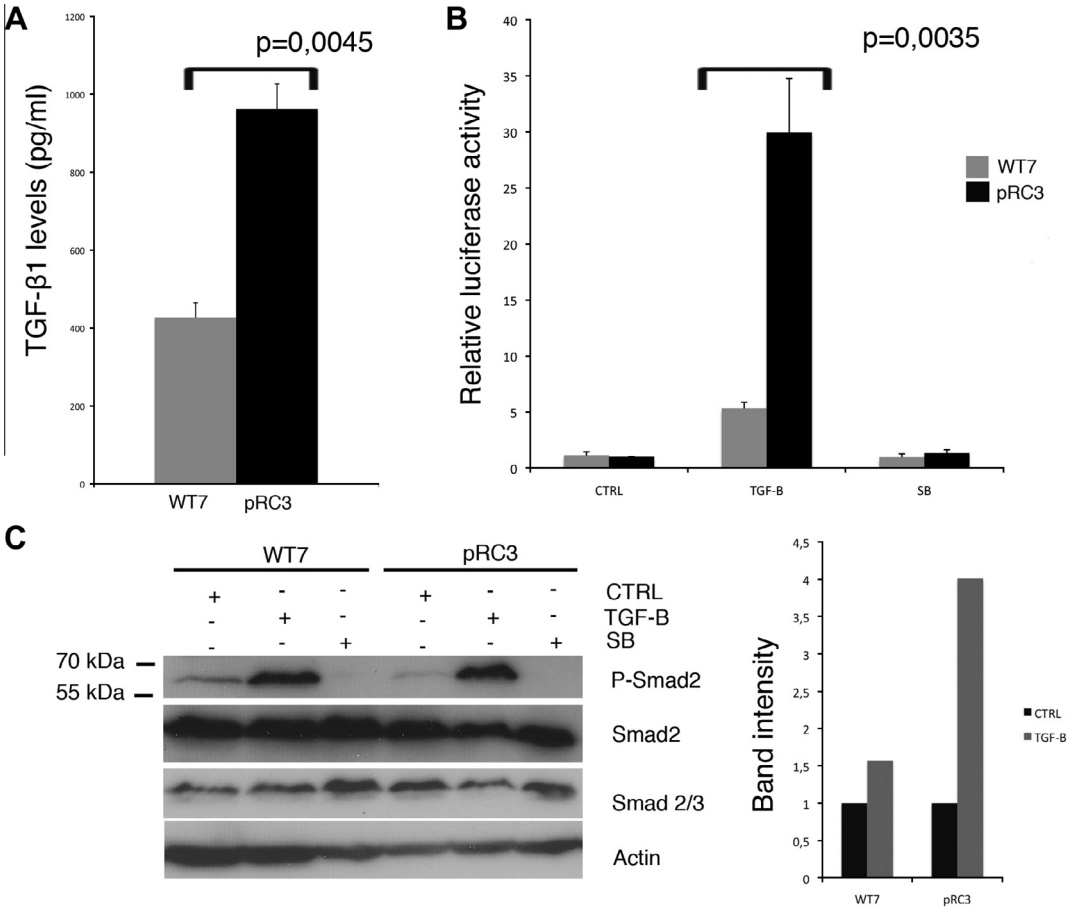

Fig. 4. (A) Quantitative determination of activated TGF-β1 concentrations in supernates from VHL reconstituted cells (WT7) and control cells (pRC3) measured by ELISA. (B) WT7 and pRC3 cells were transfected with $(CAGA)_{12}$-Luc reporter construct followed by TGF-β1, SB431542 or vehicle treatment and analyzed for relative luciferase activity. Data was normalized to control vehicle treated cells. (C) Western blot analysis of WT7 and pRC3 cells treated with TGF-β1, SB431542 or vehicle control. Protein was detected by incubation with rabbit anti-phosphorylated-Smad2 antibody (P-Smad2). The membrane was re-probed with anti-Smad2 and anti-Actin antibodies to ensure equal loading. Bar represent quantification of band intensity of P-Smad2 normalized to Actin.

with previous reports [16]. Moreover, we employed the Smad responsive luciferase reporter to study the TGF-β responsiveness in these cell lines. In comparison to control cells, the WT7 cells showed significantly lower capacity to induce the reporter activity upon TGF-β1 stimulation (Fig. 4B). When assessing the induction of P-Smad2 upon TGF-β1 stimulation both clones responded with increased phosphorylation of Smad2 (Fig. 4C). However, the induction was more pronounced in pRC3 cells as shown by quantification of the band intensities (Fig. 4C). All together these observations indicate that functional *VHL* dampens the TGF-β pathway activity in ccRCC cells and that loss of *VHL* not only enhance secretion of TGF-β1, but also enhances the responsiveness to TGF-β stimulation.

4. Discussion

Numerous studies show that TGF-β signaling contributes to the aggressiveness of ccRCC [16,30,31], however the mechanism behind the tumor promoting effects remains elusive. In the present study we wanted to further clarify the role of TGF-β signaling in this tumor type by using an integrated functional and genomic approach. In line with our previous report we could show a strong inducibility of the TGF-β pathway in ccRCC cells, as assessed by increased Smad reporter activity and increased levels of P-Smad2 in response to TGF-β1 stimulation. However, one cell line, SKRC-17, remained virtually refractory to TGF-β1 stimulation both in reporter and Western blot experiments (Fig. 1B and C). This insensitivity could relate to mutations in core components of the pathway, as reported in other tumor system [32,33].

By performing expression profiling we were able to define a 157-gene signature of the TGF-β response in ccRCC. Not surprisingly, we detected considerable similarities to previously published TGF-β gene sets derived from other cells systems, thereby validating a *bona fide* TGF-β response in ccRCC cells. In our previous study we generated a surrogate TGF-β profile from gamma-secretase treated ccRCC cells and this profile could predict poor outcome in ccRCC patients [17]. In the present paper we could verify these results, this time using the ccRCC-specific TGF-β gene signature that displayed a prognostic capacity in a ccRCC patient cohort. Thus, patients with elevated TGF-β pathway activity show worse prognosis compared to patients with tumors characterized by a low pathway activity. We also found that TβRI inhibition decreased the invasive capacity of ccRCC cells expressing basal TGF-β pathway activity, indicating that these cells to some extent rely on autocrine TGF-β activation to induce an invasive phenotype, a result well in line with the reported correlation of TGF-β pathway activity and poor clinical outcome. This observation might be linked to our studies of sarcomatoid ccRCC, which is characterized by fields of mesenchymal like cells and strongly associated with a metastatic disease. In this work we showed that high levels of TGF-β1 was detected in sarcomatoid tumor sections, and that primary

cells treated with TGF-β1 acquired a mesenchymal phenotype, thus underscoring the role of TGF-β signaling in dissemination of ccRCC [18].

Both TGF-β signaling and hypoxia have been associated with tumor aggressiveness and it has been shown that hypoxia augments TGF-β signaling through up regulation of the ligand TGF-β1 [34,35]. As a consequence, the TGF-β pathway may represent an integral part of the cellular response to chronic hypoxia. However, the cross talk between the two signaling pathways remains poorly understood. In order to approach some aspects of this cross talk we studied VHL reconstituted ccRCC cells. A significant decrease in TGF-β secretion, as well as a decreased responsiveness of Smad reporter construct in the VHL reconstituted cells was detected. Thus, the pseudo-hypoxic phenotype of ccRCC cells may render these cells more susceptible for the action of TGF-β. Moreover, our microarray analyses indicated that TGF-β stimulation lead to an enhanced expression of hypoxia regulated genes, suggesting communality between cellular responses to TGF-β and hypoxia. This could in part be explained by studies showing that some hypoxic target genes such as endoglin, VEGF and Sp1 contains binding sites for both HIF and Smad proteins allowing for synergistic effects [36–38]. Also, a stabilization of the HIF-1α protein upon activation of the TGF-β has been reported [39]. Altogether these observations indicate that the pseudo-hypoxic phenotype of ccRCC is associated with elevated TGF-β1 signaling and that the two pathways converge in their transcriptional response. Since VHL loss is intimately associated with the genesis of ccRCC this might explain the basal TGF-β activity seen in ccRCC tumors. However, our data also show that exogenous TGF-β1 can further enhance pathway activity, and in a clinical setting this might be related to secretion from tumor stroma, and be manifested in a more aggressive tumor behavior.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.bbrc.2013.04.054.

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