Wntless promotes bladder cancer growth and acts synergistically as a molecular target in combination with cisplatin

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The Wnt signaling pathway belongs to the embryonic singaling pathways that are not only essential in the developing embryo but are also dyregulated in cancer. In bladder cancer, both genetic and epigenetic alterations have been demonstrated, and activation of Wnt signaling in murine models has been linked to the development of bladder cancer. In the canonical branch of this pathway, the protein β-catenin is dephosphorylated and translocates to the nucleus, where together with TCF/LEF transcription factors, Wnt target gene expression is activated. To date, there are controversial reports on activation of this mechanism on patient material. In this study, we wanted to characterize the activation status of canonical Wnt signaling

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on a functional level to investigate its role on bladder cancer growth and develop strategies to target this pathway for therapy. Wnt proteins are transported to the cell surface by the cargo protein Wntless (WLS). We cloned its gene and generated cell lines that constitutively express WLS. Contribution of extracellular and intracellular molecular components of this pathway on Wnt activation and cell growth was examined by either recombinant overexpression or silencing by using siRNAs or lentiviral shRNA technology. Protein expression was analyzed using immunoblotting, and for cell growth, cell viability and cell cycle progression were performed. Canonical Wnt activity was measured using the TOPflash/FOPflash luciferase reporter assay. For xenograft studies, the chorioallantois membrane model (CAM) in the developing chicken emryo was used. As a major result, we observed a constitutive activation of Wnt signaling in 9 different cell lines that was controlled by the expression level of regulatory proteins within the pathway. We also characterized for the first time in bladder cancer the protein WLS that was expressed in a panel of 17 cell lines. Genetic silencing of WLS or the ectopic expression of the extracellular antagonist WIF1 resulted in diminished tumor growth in the CAM model. In combination with the standard chemotherapy drug cisplatin, silencing of WLS expression showed synergistic effects on cell growth. We conclude that the Wnt signaling pathway should be considered as a molecular target for the development of novel therapies in bladder cancer.

1. Introduction

Reactivation of embryonic/morphogenic Notch, Hedgehog, and Wnt signaling pathways is involved in tumorigenesis and tumor growth. In particular, Wnt signaling affects and maintains cancer stem cells with inherent drug-resistant properties in many tumor entities [1,2].

The family of Wnt proteins comprises 19 members of secreted molecules that are highly conserved across species. They regulate cell growth, apoptosis, motility, and differentiation during embryonic development [3]. Following palmitoylation, Wnt proteins bind in the endoplasmatic reticulum to WLS, a multispan transmembrane cargo protein that regulates transport to and release of Wnt ligands at the cell surface [4]. WLS has been described to promote proliferation of cancer cells, and its overexpression has correlated with poor clinical outcome [5]. Extracellular Wnt proteins initiate activation of the Wnt signaling pathway by binding to more than 15 cognate receptors and coreceptors on the cell surface, which comprise Frizzled 1 to 10, LRP5/6, MUSK, PTK7, ROR1/2, RYK, and also the glycosaminoglycan chains of heparin sulfate proteoglycans (HSPGs) [3]. Binding and release of Wnt ligands by HSPGs is determined by the pattern of sulfate modification, which can be modified by the extracellular sulfatases HSulf-1 and -2 [6,7]. Wnt activity is also regulated by soluble antagonists, including Wnt-inhibitory factor 1 (WIF1), secreted Frizzled-related proteins (sFRPs 1–4), or Dickkopf proteins (DKK) [2]. Activation of the canonical branch of the pathway is characterized by the dephosphorylation and accumulation of β-catenin in the cytoplasm and its subsequent translocation to the nucleus where it activates in a complex with members of the TCF/LEF family of transcription factors gene expression [2].

Although molecular alterations within the Wnt signaling pathway have been described in bladder cancer, its contribution to tumorigenesis and tumor growth is still not fully understood. Characterizing the role of this essential signaling pathway in bladder cancer might, therefore, increase understanding of the underlying biology of this tumor entity and enable development of better treatment strategies.

Epigenetic changes in hypermethylation of extracellular Wnt antagonists such as *SFRPs*, *WIF1*, or *DKK* in bladder cancer specimen compared to normal bladder mucosa or cell lines are frequent [8,9]. Diminished expression of sFRPs was associated with higher tumor stage and grade and shorter overall survival [10]. Analysis of single nucleotide polymorphisms (SNPs) in *SFRP1* and *LRP6* is associated with bladder cancer risk [11,12]. Expression of Wnt5a correlated positively with histological grade and stage [13]. However, contradictory observations have been published on the nuclear accumulation of β-catenin [14,15]. Functionally, expression of the recombinant Wnt antagonist WIF1 resulted in cell cycle arrest and growth inhibition in bladder cancer cell lines [16]. Also, activation of Wnt signaling has been demonstrated to be involved in metastasis and might contribute to chemoresistance [17,18]. In murine models, Wnt signaling is activated in a compartment of basal highly tumorigenic cells in urothelial cancer and in the regenerative proliferation of urothelial precursor cells [19,20]. Thus, although alterations in WIF1 or SFRP1/LRP affect Wnt activity and influence bladder cancer growth in vitro and murine models, observations on the relevance of molecular alterations on the activation of Wnt signaling and its contribution to tumor growth in patient specimen are controversial. The aim of this study was first to comprehensively characterize the general activation status of this pathway using a large panel of characterized cell lines of different genetic background that cover characteristics of non–muscle-invasive and muscle-invasive bladder cancer. Based on these results, we wanted to elucidate contribution of Wnt signaling to bladder cancer growth by molecular manipulation of different functional aspects of the pathway in vitro. Finally, we aimed to identify novel suitable molecular targets for therapy.

2. Material and methods

2.1. Cell lines and cell culture

Cell lines HT1376, UMUC3, J82, RT4, and T24 were obtained from the American Type Culture Collection

(VA, USA), and RT112, 647 V, BFTC905, and SW1710 from the Leibniz Institute DSMZ—German collection of microorganisms and cell cultures (Braunschweig, Germany), and EJ28 from cell lines service (Eppelheim, Germany). 253 J and 486 P were provided by Dr G. Unteregger (University of Saarland, Homburg/Saar, Germany) and 639 V, VmCUB1, SD, MGHU4 and UMUC6 were kindly provided by Dr W.A. Schulz (Heinrich-Heine-University, Düsseldorf, Germany). All cell lines were authenticated using short-tandem repeat profiling. Cells were maintained in RPMI or DMEM (Biochrom AG, Berlin, Germany) at 5% or 10% CO_2, respectively, supplemented with 10% fetal bovine serum (Biochrom AG, Berlin, Germany) and 1% NEAA (Biochrom AG, Berlin, Germany).

2.2. Polymerase chain reaction

Total RNAs were extracted from cells using mirVana miRNA isolation kit followed by a reverse transcription into cDNA with the high-capacity cDNA reverse transcription kit (Life Technology Inc, Waltham, USA) as per manufacturer's instructions. Conditions for denaturing, annealing, and extension of the template cDNA were as follows: 94°C for 30 seconds, 60°C for 30 seconds, and 72°C for 2 minutes for 30 cycles. PCR products were separated by electrophoresis on 2% agarose gels and visualized using ethidium bromide.

2.3. Luciferase reporter assays

This assay measures activity of TCF transcription factor as a read out for measuring canonical Wnt signaling. Cells were transfected with either TOPflash or FOPflash and Renilla-Luciferase plasmids (Promega Corporation, Fitchburg, USA). After 48 hours, cells were analyzed using the Dual Luciferase Reporter Assay system (Promega Corporation, Fitchburg, USA) and analyzed as described [7]. After normaliziation to the Renilla-Luciferase activity, the TOPflash signal was divided by the FOPflash signal. Results indicate the mean ± standard error of relative luminescence units from 3 independent experiments each performed in triplicates.

2.4. Plasmid, siRNA, shRNA constructs, and transfection/infection

Oligonucleotide sequences are provided in Table 1. For cloning of WLS, a full-length PCR amplificate (primer pair WLS1F/WLS1R) was purified via gel extraction (QIAquick Gel Extraction Kit, Qiagen, Oslo, Norway), cloned into the plasmid pcDNA3.1/V5-His-TOPO (Life Technologies, Waltham, USA), and verified by sequencing. For transfection of cells, FuGENE HD (Promega Corporation, Fitchburg, USA) or Lipofectamine RNAimax (Life Technologies, Waltham, USA) was used according to the manufacturer's instructions. For siRNA delivery, 10 nanomolar of stealth RNAi oligonucleotides (Life Technologies, Waltham, USA) was used. All assays were conducted 48 or 72 hours after transfection. For infections with lentiviral vectors, conditions were applied as described previously [7]. DKK1 and WIF1 expression vectors were kindly provided by Dr Steven D. Rosen [7].

Table
List of oligonucleotides

Target	Direction	Sequence
Oligonucleotides for RT-PCR		
Wnt-2b	Forward	AGT CTT CGG GGA GCT ATG CT
	Reverse	GGT CTG GTC CAG CCA CTC T
Wnt-4	Forward	TCA GAG CAT CCT GAC CAC TG
	Reverse	GCA TCT CAG AGG AGG AGA CG
Wnt-7a	Forward	GGG ACT ATG AAC CGG AAA GC
	Reverse	TGC GGA ACT GAA ACT GAC AC
LRP-5	Forward	CCG GAA GAT CAT TGT GGA CT
	Reverse	GTG TAG AAA GGC TCG CTT GG
LRP-6	Forward	ATG TCA GCG AAG AAG CCA TT
	Reverse	AAC GTC AAG GCA AAA GGA TG
TCF-1	Forward	GAG CAA AGA GGC ACT GAT CC
	Reverse	GAG TGC CCT TGT TGA GGT GT
TCF-3	Forward	GTT CGG AGG TTC AGG TCT TG
	Reverse	GGT ACA CCG AGG ATG GAA GA
TCF-4	Forward	TTC ACC TCC TGT GAG CAG TG
	Reverse	GTG CTT GCT GAT GGA GCA TA
LEF-1	Forward	CCT GGA GAA AAG TGC TCG TC
	Reverse	GAC GAG ATG ATC CCC TTC AA
HSulf-2	Forward	GAG GAT TCA GCC CCA CAA TA
	Reverse	TAG AAC GGG ACC CTG ATG TC
WLS	Forward	CGT TTG CTG AGT GGA CTG AA
	Reverse	AAA CCA CAC CTT GGT GAA GC
WLS1F	Forward	AAA TGG CTG GGG CAA TTA TAG
WLS1R	Reverse	AAT ACC AGA AGC TGC GTT GTC
β-Actin	Forward	ATC TGG CAC CAC ACT TCT ACA ATG AGC TGC G
	Reverse	GTC ATA CTC CTG CTT GCT GAT CCA CAT CTG C
siRNA oligonucleotides		
β-Catenin	F1	AGCUGAUAUUGAUGGACAG
	R1	CUGUCCAUCAAUAUCAGCU
	F2	CAGUUGUGGUUAAGCUCUU
	R2	AAGAGCUUAACCACAACUG
Wls	F1	UCCAGAUACUGUAGAAGGGAUUCGU
	R1	ACGAAUCCCUUCUACAGUAUCUGGA
	F2	ACCAACCGGAUAUCCUUUAUCUCCC
	R2	GGGAGAUAAAGGAUAUCCGGUUGGU
ctrl	F	AACGUACGCGGAAUACAACGA
	R	UCGUUGUAUUCCGCGUACGUU

2.5. Cell viability assay and combination index calculation

CellTiter-Blue cell viability assay (Promega Corporation, Fitchburg, USA) was performed in triplicates according to the manufacturer's protocol and analyzed using the VictorX3 Multilabel plate reader (PerkinElmer, Waltham, USA), and the standard error was calculated. Asterisk means Student's *t*-test, $P < 0.05$. All experiments were done at least in 3 independent replicates. The combination index was calculated using CompuSyn (Combo Syn Inc, Paramus, NJ) according to the Chou-Talalay method [21].

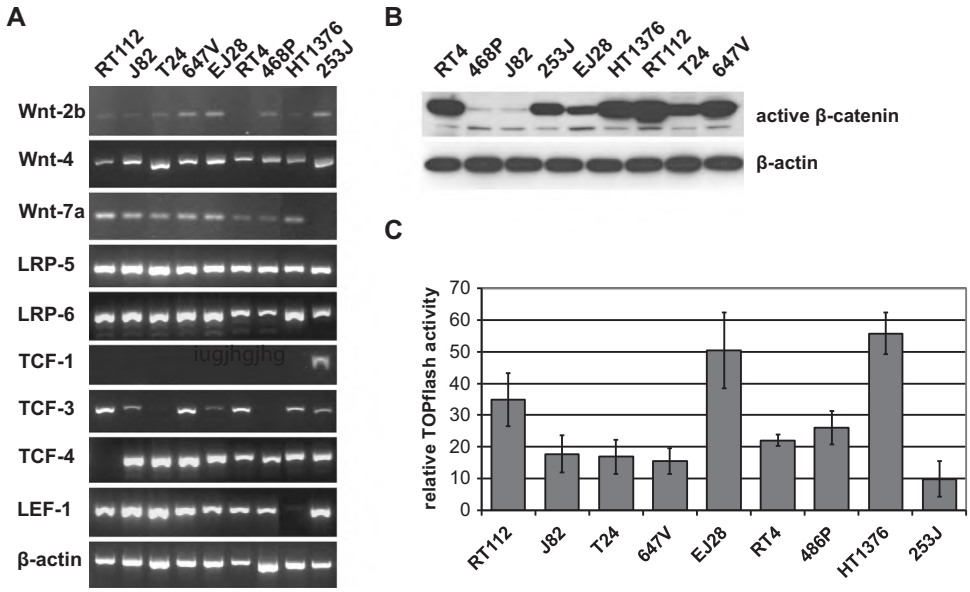


Fig. 1. Expression of molecular components and activation level of the Wnt signaling pathway in bladder cancer. Transcripts of depicted genes were detected by using RT-PCR on different cell lines (A). Total cell lysates were subjected to SDS-PAGE, and immunoblots were performed for nonphosphorylated β-catenin or β-actin as loading control (B). (C) Cells were transfected with the TOPflash/FOPflash reporter system and 2 days later analyzed. Light units were normalized to the renilla luciferase signal, and the firefly luciferase signal of the TOPflash was divided by the background signal of the FOPflash reporter plasmid. SDS-PAGE = sodium dodecyl sulfate polyacrylamide gel electrophoresis.

2.6. Cell cycle progression

Two days after transfection with plasmid or siRNA constructs or after infection with lentiviral shRNA, cells were pulsed for 2 hours with BrdU, using the APC BrdU Flow Kit (BD Biosciences, Franklin Lakes, USA), according to the manufacturer's protocol. BrdU/APC staining was measured by flow cytometry.

2.7. Immunoblotting

Immunoblotting was performed as described previously [22]. Antibodies against following antigens were used: active β-Catenin, V5-tag, WLS (Millipore, Temecula, USA), β-catenin (BD Bioscience, Franklin Lakes, USA), β-Actin (Sigma, St. Louis, USA), GSK3β, pGSK3β, (Cell Signaling, Danvers, USA), and HSulf-2 (provided by Prof Stephen D. Rosen, UCSF, San Francisco, USA). HRPO conjugated secondary antibodies were purchased from Dianova (Hamburg, Germany). Amersham ECL Prime Western blotting reagent (GE Healthcare, Little Chalfont, UK) was used for detection.

2.8. Chicken chorioallantoic membrane (CAM) assay

The CAM assay was performed as described previously by our group, using fertilized white Leghorn chicken eggs (Brüterei Süd, Regenstauf, Germany) [22]. Tumor weights are depicted with boxplots where the horizontal line indicates the median, upper whisker the difference between maximum and first quartile, and the lower whisker indicates difference between minimum and third quartile. Asterisks indicate Student's *t*-test with $P < 0.05$.

3. Results

3.1. Expression and activation status of molecules in the Wnt signaling pathway in bladder cancer

We first analyzed transcription of Wnt ligands Wnt-2b, -4, -7a, the receptors LRP5/6, and the transcription factors TCF-1, -3, -4, and LEF in 9 bladder cancer cell lines by reverse transcription polymerase chain reaction (RT-PCR) (Fig. 1A). At least 1 Wnt ligand, LRP, TCF, and LEF could be detected in all cell lines. As an indicator for downstream activation of the pathway, protein level of nonphosphorylated β-catenin on Ser37/Thr41 was assessed by Western blot, showing strong expression in 7 of 9 and weak expression in 2 cell lines (Fig. 1B). We next assessed the relative activation level of canonical Wnt signaling in these cell lines by using the TOPflash/FOPflash reporter assay. All cell lines showed a positive signal, indicating activation of the intranuclear β-catenin/TCF/LEF complex (Fig. 1C).

We also performed a general analysis with 52 genes on 127 bladder cancer specimen in the TCGA Research Network database (http://cancergenome.nih.gov/) including Wnt ligands, FRZ receptors, all extracellular inhibitors, HSulf1/2, and intracellular molecules such as APC, AXIN1, GSK3B, CTNNB, DVL1, DVL2, and DVL3. In 64% of tumors, molecular alterations could be observed, but overall

survival was statistically not significantly affected (data not shown).

3.2. Functional analysis of Wnt pathway regulation

Next, we examined the influence of intracellular and extracellular molecular regulators on the activity of the canonical Wnt pathway enabling the TOPflash/FOPflash reporter system. First, we tested the influence of β-catenin expression level on Wnt activation by silencing its expression using 2 independent siRNA oligonucleotides (Fig. 2A). Silencing of β-catenin expression correlated with a decrease of Wnt activity by 30% to 60% in RT112, T24, J82, and 647 V cells (Fig. 2B). We then transfected cells with the recombinant extracellular Wnt inhibitors WIF1 and DKK. In Western blot analysis, expression of either proteins resulted in the down-regulation of active β-catenin and increased phosphorylation of GSK3β indicating inactivation of the Wnt pathway (Fig. 2C). Overall expression of β-catenin and GSK3β was unchanged. Also in the TOPFlash/FOPFlash assay, expression of either WNT inhibitor induced a 40% to 50% decrease in Wnt-activity in 4 different cell lines (Fig. 2D). We additionally examined the influence of the sulfation pattern of heparin sulfate glucosaminglycan (HSPG) chains by modulating expression of HSulf-2 [3]. We first assessed HSulf-2 expression by using a semiquantitative RT-PCR, showing that it was expressed in 5 of 9 cell lines (Fig. 2E). For further analysis, we reduced expression of HSulf-2 in RT112 cells by a lentiviral shRNA approach (Fig. 2E, lower panel). Wnt activity decreased by 25% to 30% indicating a positive regulatory role of HSulf-2 on Wnt activity in bladder cancer (Fig. 2F).

3.3. WLS is expressed in bladder cancer and positively regulates Wnt signaling

Because of its potential as a novel therapeutic target, we analyzed WLS in greater detail. Its expression was confirmed by RT-PCR in 17 (Fig. 3A) and by Western blotting in 6 selected bladder cancer cell lines. In the RT112 cells, a relatively low expression level of WLS mRNA correlated with a lower protein level (Fig. 3B). Next, we cloned the WLS gene and confirmed protein expression by transfecting Chinese hamster ovary cells with this recombinant construct and performance of immunoblotting against the fused V5-His tag (Fig. 3C). Ectopic expression of this recombinant WLS significantly ($P < 0.01$) increased Wnt activity in 4 cell lines by 1.5- to 2-fold (Fig. 3D). When analyzing molecular alterations described in the TCGA Research Network, 8% of the 127 examined specimen exhibit amplifications or mRNA up-regulation of WLS, whereas in 2%, mutations could be observed without affecting statistically significant overall survival (data not shown).

To examine WLS dependent Wnt activity, we established RT112 cells stably transfected with WLS because of their relative low level of endogenous WLS and T24 cells (RT112-WLS, T24-WLS, Fig. 4A), selecting 3 independent clones each. We also established 3 independent siRNAs (WLSsiRNA1–3) that specifically silence expression of WLS (Fig. 4B). Silencing of WLS expression in parental T24 and RT112-WLS cells resulted in a decrease of the TOPflash reporter assay ($P < 0.05$), whereas the parental RT112 with lower endogenous WLS protein level did not show significant changes (Fig. 4C). In parallel, we showed reduced cell viability corresponding to the WLS-regulated Wnt activation status of these cells (Fig. 4D). When testing WLS expression on cell cycle progression, we could demonstrate that different T24-WLS clones did display accumulation of cells in S-phase compared to parental T24 cells (Fig. 4E). To further examine WLS-dependent availability of extracellular Wnt ligands, we transfected RT112-WLS cells with the extracellular Wnt antagonists WIF1 and DKK that interfere with binding of Wnt ligands to their cognate receptors. Expression of both proteins was confirmed by Western blotting (data not shown) and resulted in a 50% decrease of Wnt activity (Fig. 4F).

3.4. Modulation of Wnt activity strongly affects tumor growth

Finally, we analyzed effects of WLS expression and its combination with chemotherapy on tumor growth. For this approach, we used the 3-dimensional xenograft model of the CAM [23]. RT112-WLS cells were transfected with either recombinant WIF1, WLS siRNA, or control siRNA in culture and transplanted on the CAM. After 8 days, tumors were harvested, trimmed, and weighed. Tumors were formed in all 3 conditions but were significantly reduced in weight following treatment with WLS siRNA (40%) and WIF (50%) expressing tumors (Fig. 5A, $P < 0.001$). We next used T24-WLS cells and silenced WLS using siRNAs (Fig. 5B) or expressed recombinant WLS in parental T24 cells (Fig. 5C). Again, silencing of WLS reduced tumor weight by 61% ($P < 0.01$), whereas expression of recombinant WLS induced tumor growth by 167% ($P < 0.001$). To further investigate suitable improved treatment strategies, we analyzed the combination of WLS silencing with the standard chemotherapy drug cisplatin on cellular growth. T24 cells were transfected with WLS- or control siRNA and treated with different concentrations of cisplatin over a period of 72 hours. Cell viability was assessed, and the combination index was calculated that resulted in values lower than 1 for the 3 concentrations tested, indicating synergism when combining these 2 treatment strategies (Fig. 5D and E).

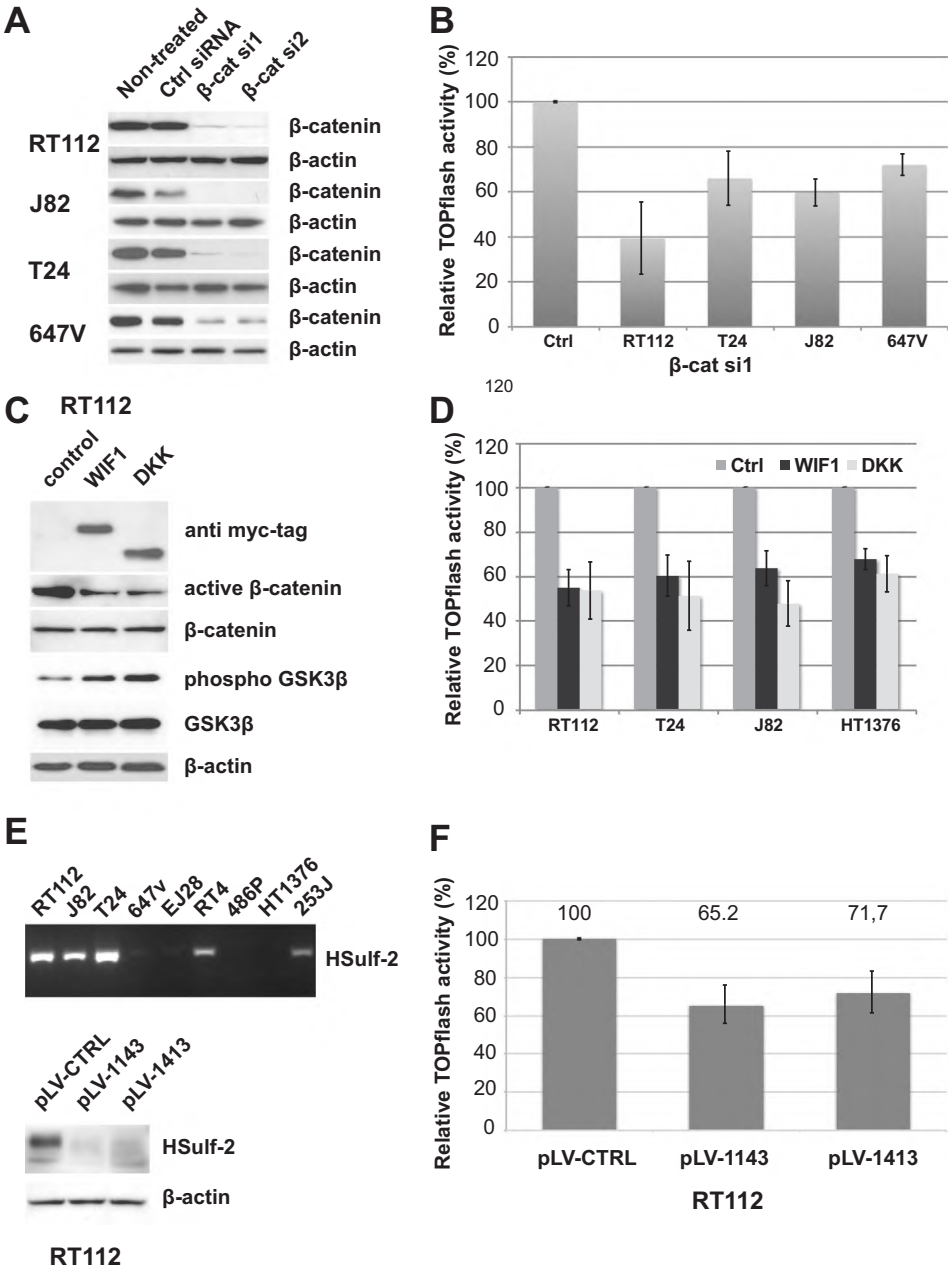


Fig. 2. Regulation of Wnt activity in bladder cancer cells. (A) Silencing of β-catenin 2 days after transfection of cells with control, or 2 different siRNAs was detected by immunoblotting. (B) Two days after transfection with control and β-catenin-specific siRNAs in combination with the TOP/FOPflash system, cells were analyzed for reporter activity. For direct comparison of the 4 cell lines, the control of each cell line was set to 100%. Bars represent the combined values of both independent siRNAs used. (C) Cells were transfected with recombinant WIF1 or DKK 2 days after transfection lysed. After protein separation by SDS-PAGE, immunoblots against phospho- and total β-catenin and GSK3β were performed. (D) The effect of Wnt inhibitors WIF1 and DKK on the TOP/FOPflash reporter activity was analyzed 2 days after transfection. (E) RT-PCR in different cell lines to detect expression of HSulf-2 (upper panel) and immunoblot for HSulf-2 two days after transduction with control and 2 different shRNAs and β-actin as loading control. (F) TOP/FOPflash reporter activity 2 days after transduction/transfection of cells. Changes in (B), (D), and (F) relative to control were statistically significant ($P < 0.05$). SDS-PAGE = sodium dodecyl sulfate polyacrylamide gel electrophoresis.

4. Discussion

In this study, we analyzed the activation status of the Wnt signaling pathway in bladder cancer cell lines and identified the WLS protein as a potential novel molecular target for therapy. We could demonstrate constitutive activation of the Wnt pathway in a panel of 9 cell lines. It is of interest that in our data, expression level of single molecular elements in the pathway (e.g., β-catenin in 468 P and J82 cells, Fig. 2B or WLS in RT112, Fig. 3A) do not directly correlate with the activation status of the TCF/LEF reporter system. Importantly, manipulation of these cell lines with extracellular Wnt inhibitors (WIF1, DKK1, or HSulf-1 silencing) regulates activity of the TCF/LEF

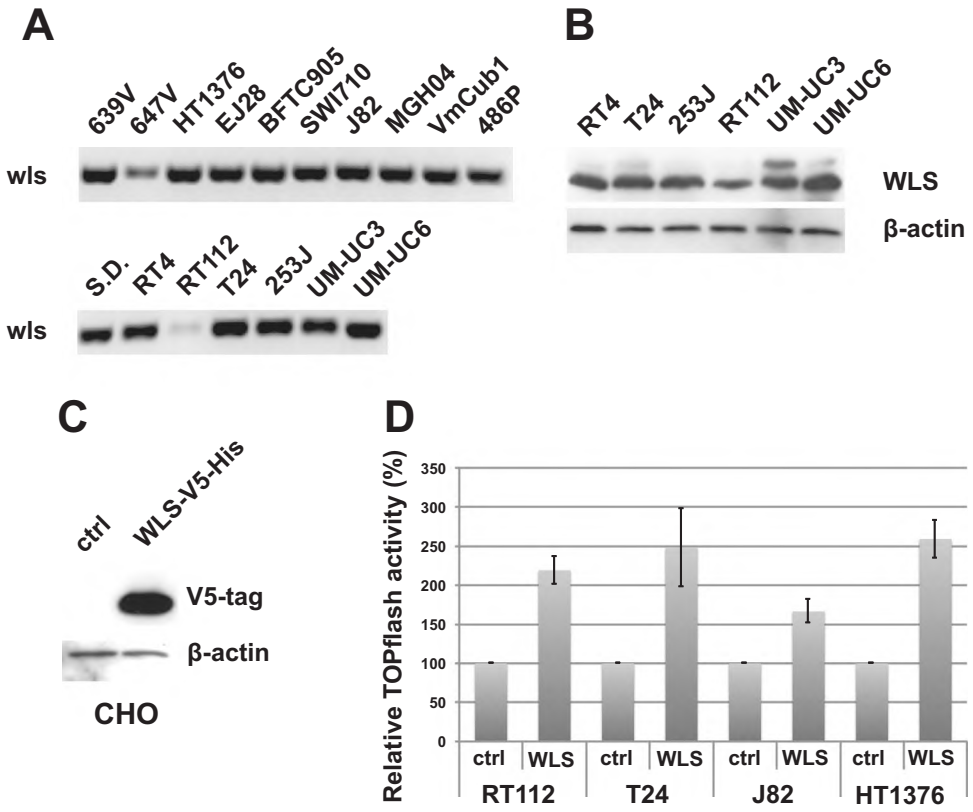


Fig. 3. Bladder cancer cell lines express WLS, which is involved in regulating Wnt activity. Expression of WLS transcript was analyzed in a panel of 17 blader cancer cell lines by RT-PCR (A). Protein expression of WLS was confirmed in a panel of 6 different bladder cancer cell lines by performing immunoblots against WLS and as a loading control β-actin on whole cell lysates (B). CHO cells were transfected with either empty vector (ctrl) or WLS-V5-His, and expression of the recombinant WLS was analyzed on whole cell lysates 2 days after transfection by immunoblotting, using *n* antibody against the V5-tag (C). (D) Effects of the recombinant WLS on Wnt activity was tested using the TOP/FOPflash reporter assay 2 days after transfection of cells. CHO = Chinese hamster ovary.

reporter indicating that the absolute expression level of single molecular components might not be the only decisive element in the control of this pathway.

Activation of Wnt signaling has been reported before, for example, in T24, RT4, J82, and SW480 bladder cancer cell lines [13,16,18,24]. As for RT112, Wnt activity has been reported to be very low, and the response when transfected with sFRP1 was not significant [24]. However, most cell lines did respond in a decrease in Wnt activity when infected with extracellular inhibitors WIF1 or long non-coding RNAs that influence Wnt expression.

A contribution of the Wnt pathway to bladder cancer tumorigenesis has been demonstrated in murine transgenic models. Expression of activated β-catenin resulted in the spontaneous formation of hyperproliferative lesions [25]. However, only when combining this genetic alteration with the deletion of PTEN, urothelial carcinoma (UC) developed. Also, mice bearing mutations in H-Ras or K-Ras developed UC only in combination with β-catenin activation [26]. Interestingly, nuclear localization of β-catenin has been demonstrated in the N-butyl-N-(-4-hydroxybutyl)nitrosamine-induced murine bladder cancer model [27]. These studies show that activation of the canonical branch of the Wnt signaling pathway can contribute to tumorigenesis of the bladder. However, analysis of available data sets of patient specimen in the TCGA cohort does not support the occurance of mutations in the pathway that clearly affect tumor growth. Thus, given the body of evidence from preclinical studies, we hypothesize that rather than by mutations its activation in bladder cancer may usually be brought about by changes in the expression and availability of WNT ligands and antagonists that could influence by autocrine mechanisms of tumor growth.

In vitro, expression of WIF1 influences cell proliferation and tumor growth, whereas no effects for sFRP1 could be observed [16,24]. We extended these results by using a larger panel of cell lines and analysis of a larger range of molecules including cell surface receptors and intracellular components for their expression and influence on pathway activity. In keeping with previous reports, recombinant expression of the Wnt antagonist WIF1 negatively regulated Wnt activity [16]. We extended these data by interfering with DKK, β-catenin, and HSulf-2 protein level that also resulted in the regulation of Wnt activity, indicating a fully activatable Wnt signaling pathway in these cell lines. We also examined for the first time the expression and function of WLS in bladder cancer. Our data suggest that WLS expression increases the availability of active Wnt ligands at

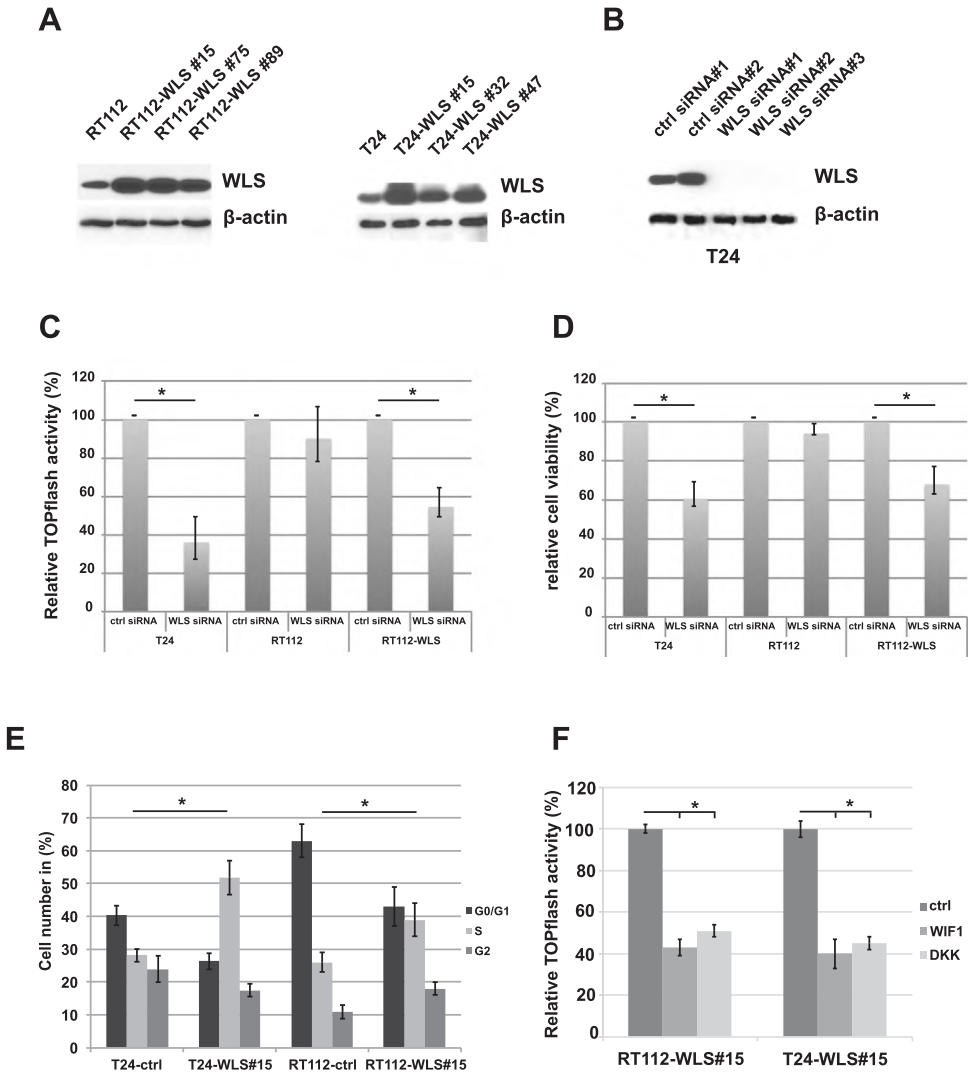
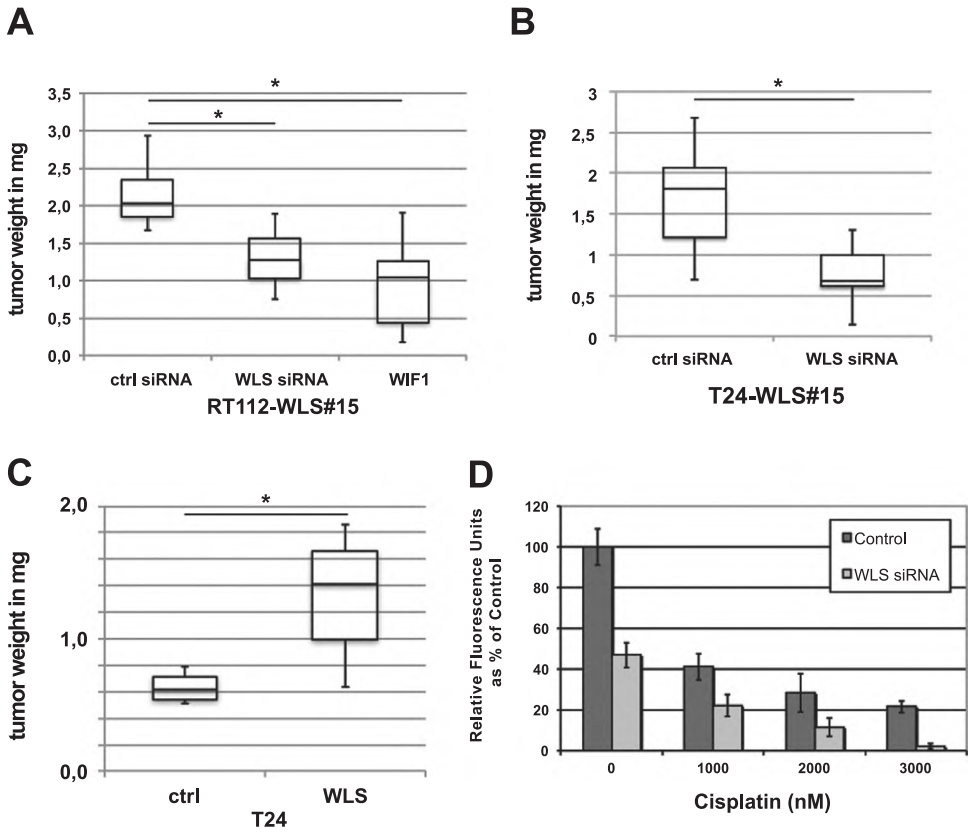


Fig. 4. WLS expression level regulates Wnt activity, cell viability, and cell cycle progression. (A) RT112 and T24 cells were transfected with the WLS expression plasmid, selected, and single clones were analyzed for protein expression in Western blots using whole cell lysates. (B) T24 cells were transfected with different siRNAs directed against WLS, and their efficiency was analyzed in Western blots. Effect of WLS silencing on TOPflash activity (C) or cell viability (D) was analyzed in T24, RT112, and RT112-WLS15 two days after transfection of cells with WLS siRNA1. The effect of WLS overexpression on cell cycle progression was analyzed in 3 stable WLS overexpressing T24 cell clones relative to a control, using BrdU incorporation and staining with APC (E). RT112-WLS15 cells were transfected with the extracellular Wnt inhibitors WIF1 and DKK, and TOPflash activity was measured 2 days after transfection (F). Statistical analysis was performed by the Student's *t*-test and $P < 0.005$ (*).

the cell surface thereby activating the pathway and promoting cell cycle progression.

In melanoma, WLS functions as a negative regulator of cell proliferation and spontaneous metastasis by activating WNT/β-catenin signaling [28]. Our observations show that inactivation of WLS does partially suppress Wnt activity and regulate cell cycle progression, cell viability, and tumor growth in the CAM model. It is probably not feasible to completely block the Wnt signaling pathway owing to severe expected toxicities to the patient. As we have shown, the partial influence of WLS on this pathway in combination with established chemotherapies such as cisplatin might be a suitable novel therapy approach. Activation of Wnt signaling as a cellular response mechanism to chemotherapy has been described before [29–31]. The molecular mechanism that is involved in this chemoresistance includes, in all studies, the activation of the canonical branch of the Wnt signaling pathway by activation of different molecules. Thus, the synergistic effect when combining WLS inhibition with cisplatin is probably based on the partial suppression of Wnt activity.

A growing number of small molecules with inhibitory effects on Wnt signaling are in preclinical development [32]. Pharmacological inhibitors of Wnt signaling have been developed to target this complex pathway at different sites [2]. However, a major challenge still is a direct targeting of the pathway owing to the redundancy of many molecular components in the pathway. Also, data from some clinical trials suggest that targeting of Wnt signaling as a universal approach might not be suitable



E

Cisplatin (nM)	Combination Index of cisplatin and WLS siRNA combination
1000	0,82803
2000	0,56535
3000	0,12689

Fig. 5. Tumor growth is influenced by WLS expression level. (A) RT112-WLS cells were transfected with siRNAs against WLS or with WIF1 and seeded on the CAM. Eight days later, tumor weight was measured. The T24-WLS15 cell clone was transfected with WLS siRNA, (B) and parental T24 cells were transfected with the expression plasmid encoding for WLS (C). Cells were grown as 3 dimensional xenografts, and tumor weight was measured from at least 8 independent tumors. Statistical analysis was performed by Student's *t*-test and $P < 0.005$ (*). (D) T24 cells were transfected with WLS siRNA or control siRNA and 1 day later treated with different concentrations of cisplatin for another 2 days. Effects on cell viability were measured using the Cell-Titer-Blue assay, and the combination index according to the Chou-Talalay method was calculated (E). Values lower than 1 mean synergism.

owing to on-target side effects in the stem cell niche that can cause adverse effects, for example, on skeletal constitution [33]. Although there is no dedicated WLS inhibitor, modulators of vesicular pH such as Bafilomycin also suppress WLS activity [34]. Those data show that this molecule can be addressed as a direct drug target.

5. Conclusion

Canonical Wnt signaling is constitutively activated in bladder cancer cell lines and is modulated by intracellular and extracellular regulatory molecules. The cargo receptor WLS is involved in the activation of Wnt signaling and tumor growth. For therapy, down-regulation of WLS expression in combination with cisplatin acts synergistically.

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