Vascular Endothelial Growth Factor Receptor-1 Is Synthetic Lethal to Aberrant β-Catenin Activation in Colon Cancer
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Abstract

Purpose: The Wnt/β-catenin (β-cat) signaling cascade is a key regulator of development, and dysregulation of Wnt/β-cat contributes to selected cancers, such as colorectal, breast, and hepatocellular carcinoma, through abnormal activation of Wnt target genes. To identify novel modulators of the Wnt/β-cat pathway that may emerge as therapeutic targets, we did an unbiased high-throughput RNA interference screen.

Experimental Design: A synthetic oligonucleotide small interfering RNA library targeting 691 known and predicted human kinases was screened in Wnt3a-stimulated human cells in a live cell luciferase assay for modulation of Wnt/β-cat–dependent transcription. Follow-up studies of a selected high-confidence “hit” were conducted.

Results: A robust quartile-based statistical analysis and secondary screen yielded several kinases worthy of further investigation, including Cdc2L1, Lmtk3, Pank2, ErbB3, and, of note, vascular endothelial growth factor receptor (VEGFR)1/2, a receptor tyrosine kinase (TK) with putative weak kinase activity conventionally believed to be a negative regulator of angiogenesis. A series of loss-of-function, genetic null, and VEGFR TK inhibitor assays further revealed that VEGFR1 is a positive regulator of Wnt signaling that functions in a glycosynthe kinase-3β (GSK3β)–independent manner as a potential synthetic lethal target in Wnt/β-cat–addicted colon carcinoma cells.

Conclusions: This unanticipated non-endothelial link between VEGFR1 TK activity and Wnt/β-cat signaling may refine our understanding of aberrant Wnt signaling in colon carcinoma and points to new combinatorial therapeutics targeted to the tumor cell compartment, rather than angiogenesis, in the context of colon cancer. (Clin Cancer Res 2009;15(24):7529–37)

The Wnt signal transduction cascade relays progrowth signals through β-catenin (β-cat), a key transcriptional coactivator whose cellular levels are tightly regulated. In nonactive states, serine/threonine phosphorylation of β-cat by glycogen synthase kinase-3β (GSK3β), the component kinase of the adenosomatous polyposis coli–mediated destruction complex, results in β-cat polyubiquitination mediated by the E3 ligase β-TrCP and consequent proteasomal degradation. In the presence of activating Wnt ligands, the destruction complex is inhibited, and an active β-cat population accumulates in the cytoplasm and translocates into the nucleus, where it forms a transactivator complex with lymphoid-enhancing factor or T-cell factor, resulting in the transcription of >50 target genes (1, 2). Aberrant activation of the Wnt pathway resulting in ligand-independent signaling and constitutively high levels of Wnt target gene expression is common in colon cancers (1, 2). Although such Wnt/β-cat activation contributes to certain forms of cancer, it may also create therapeutically exploitable vulnerabilities within the context of synthetic lethality (3, 4). Two genes are classically synthetic lethal if cells with a mutation in either one of the genes are viable, but simultaneous mutation of both genes causes death or impairs cellular fitness (5, 6). The idea can be extended to common mechanisms of cancer transformation, such as gain-of-function oncogenes exemplified by aberrant Wnt/β-cat activity. In this regard, inhibition or mutation of a second gene may be lethal or antiproliferative only in the context of specific oncogenic upregulation (3). Thus, it may be possible to use the contextual difference of aberrant Wnt/β-cat activation to find selective targets for therapeutic intervention. Kinases, an important family of functional regulators, have emerged as therapeutically alluring targets because single kinase inhibition can often eliminate an entire stimulatory or inhibitory arm of a signal transduction cascade. We have generated a screening strategy...
Translational Relevance

Activation of Wnt signaling, which is observed constitutively in most colon cancers, targets >50 genes by a transactivator complex comprising β-catenin (β-cat) and T-cell factor. To uncover novel kinases that modulate Wnt signal transduction and are essential to the survival of cells addicted to Wnt/β-cat signaling, we systematically inactivated, one at a time, all human genes that direct the synthesis of kinases in cells stimulated with Wnt. Our screen identified an unanticipated mode of cross-talk between vascular endothelial growth factor receptor (VEGFR)1/Flt1 and the Wnt/β-cat pathway, two pathways of tremendous importance in cancer. Initial mechanistic studies provided evidence for VEGFR1/Flt1-linked tyrosine phosphorylation of β-cat, thereby modifying transcriptional activity. Thus, a mechanistically rational strategy for synthetic lethal therapy in the treatment of colon carcinoma is targeted suppression of VEGFR1-linked tyrosine kinase activity within the Wnt/β-cat–addicted tumor cell compartment per se, not endothelia.

Materials and Methods

Cell lines and reagents. pCMV-FLuc has been previously described (7). pTOPFLASH and pcDNSA.1 were purchased from Upstate Products (Billerica, MA) and Invitrogen Corporation (Carlsbad, CA), respectively. STF293 reporter cells were kindly provided by Dr. Jeremy Nathans (Johns Hopkins University School of Medicine, Baltimore, MD) and cultured in STF293 medium. HEK293, HEK293T, and HeLa cells (ATCC, Manassas, VA) were cultured in DMEM supplemented with 10% heat-inactivated fetal bovine serum and 1% glutamine. SW480 and KM12L1a colon carcinoma cells (kindly provided by Dr. Loren Michel, Washington University School of Medicine, St. Louis, MO) were cultured in DMEM supplemented with 10% heat-inactivated fetal bovine serum and 2% glutamine. 293 cells and L cells were purchased from ATCC, and conditioned medium was collected according to the ATCC protocol. A HEK293 cell line stably expressing FLuc (293Luc) was established by transfecting 293 cells with pCMV-FLuc with the use of FuGENE 6 transfection reagent (Roche, Indianapolis, IN) for 48 h followed by selection of single colonies after continuous exposure to G418 (0.4 mg/mL). SB216763 (Sigma-Aldrich, St. Louis, MO), and VEGFR tyrosine kinase (TK) inhibitors II and III (EMD Biosciences, Gibbstown, NJ) were reconstituted in DMSO. Plasmids expressing short hairpin RNA (shRNA) sequences were provided by the Genome Sequencing Center, Washington University School of Medicine. Each sequence was provided in a pLKO.1 expression vector.

High-throughput screening. Small interfering RNA (siRNA) screening was done in black, clear-bottomed, 96-well culture plates (Corning, NY) in STF293 medium (DMEM/F12 supplemented with 10% heat-inactivated fetal bovine serum and 1% glutamine) at 100 μL well (for 96-well plates) or 200 μL (for 24-well plates) or 500 μL (for 24-well plates) or 50 μL (for 96-well plates) of serum-free DMEM/F12 media. Transfection was reagent in the serum-free medium and incubated for 5min, followed by addition of DNA. After incubation for 20min, the transfection mixture was added dropoutse of wells of assay plates. Cells were incubated in the transfection mixture for 4 h, and then the media was replaced with fresh DMEM/F12 media containing serum. After transfection, cells were incubated for 24h followed by doubling of the volume in each well plus addition of Wnt3a-conditioned media that contained 150 μg/mL of Tβ-Luciferin. Cells were incubated for another 24h and then imaged on a bionanescence imaging system (VIS 100, Caliper Life Sciences, Hopkinton, MA; filter, open; Fstop, 1; exposure, 5 min; binning, 8). After bioluminescence imaging, an MTS cell viability assay was done (CellTiter 96 AQueous One Solution Cell Proliferation Assay, Promega, Madison, WI) by measuring the formation of the formazan product (absorbance at 490 nm, 1 h, 37°C) with the use of a Fluostar Optima plate reader for 24-well plates or an EL 311 plate reader (BioTek Instruments) for 96-well plates.

RT Profiler PCR Wnt Array. Embryonic stem cells from wild-type R1 or VEGFR1/Flt1+/− mice were provided by Dr. Guo-Hua Fong (University of Connecticut Health Center, Farmington, CT) and Dr. Kyunghee Choi (Washington University School of Medicine), and maintained at the Murine Embryonic Stem Cell Core at Washington University. Cells were cultured in DMEM containing FCS, L-glutamine, nonessential amino acids, HEPES, leukemia inhibitory factor, and 2-mercaptoethanol, and plated on gelatinized 10-cm tissue culture dishes to eliminate mouse embryonic fibroblasts before experimental assays. After incubation of embryonic stem cells in the presence or absence of Wnt3a, RNA was obtained with the use of the RNeasy RNA extraction kit (Qiagen), including the optional DNase digestion protocol. Reverse transcriptase-PCR array profiling was done with the use of the pathway-specific RT Profiler PCR Array system (SuperArray, Frederick, MD).
according to the manufacturer’s instruction, in an ABI 7300 real-time PCR system (Applied Biosystems, Foster City, CA). Data analysis with the use of the ΔCt method was done according to a data analysis template available online. Briefly, ΔCt values were calculated by subtracting the average cycle threshold (Ct) values for housekeeping genes (glyceraldehyde-3-phosphate dehydrogenase, β-actin, and Hprt1) from the Ct values for T (Brachyury). Fold differences were calculated as 2ΔCt (untreated)/2ΔCt (untreated) for both wild-type and VEGFR1(+/-) embryonic stem cells.

**Semiquantitative reverse transcriptase-PCR.** siRNA transfections were done as described above and allowed to proceed for 48 h. RNA was extracted with the use of a RNeasy RNA extraction kit (Qiagen). One-step reverse transcriptase-PCR was done according to the manufacturer's protocol with the use of the Access RT-PCR system (Promega) on a GeneAmp PCR system 2700 (Applied Biosystems). The primer sequences were as follows: VEGFR1 extracellular domain, forward primer TGGGACAGTGAAGACCTGTT, reverse primer GGTCGAGTCAAACACGACAA; glyceraldehyde-3-phosphate dehydrogenase, forward primer GTGAAGGTCTGAGAAGTCC, reverse primer TGTAGCAGAAGTCCCTCCCT. Amplified products were subjected to agarose gel electrophoresis, and visualized as a 384-bp product for VEGFR1 and a 200-bp product for glyceraldehyde-3-phosphate dehydrogenase with the use of a Chemilimager (Alpha Innotech Corp., San Leandro, CA).

In cellulo transfection assays. In STF293 cells, follow-up siRNA validation studies were done with the use of a forward transfection protocol with DharmaFECT 1 reagent (Dharmacon Research Inc.) at a final concentration of 50 nmol/L siRNA (ON-TARGETplus SMARTpool, L-005316-00; Dharmacon Research Inc.), and 20 nmol/L siRNA (individual duplexes, Dharmacon Research Inc.). In other experiments, cells were transfected with pTOPFLASH with the use of Lipofectamine 2000 (Invitrogen) according to the manufacturer's instructions. Bioluminescence was captured with the use of an IVIS 100 or IVIS 50 imaging system, and Living Image (Caliper Life Sciences) and Igor (WaveMetrics) software. A similar SDS-PAGE and immunoblotting protocol was followed for detection of β-caten (1:200 dilution), VEGFR1 (1:200 dilution), and actin (1:200 dilution) with the use of antibodies from Santa Cruz (Santa Cruz, CA), Chemicon Corporation (Billerica, MA), and Sigma-Aldrich, respectively.

**Statistical analysis and hit selection.** In the primary siRNA screen, the luminescence data acquired 18 h post Wnt3a treatment were normalized to cell viability data (expressed as value = ×). Median (Q2), first (Q1), and third (Q3) quartile values were calculated for all normalized values and subjected to plate-by-plate analysis. To facilitate experiment-wide analysis, further normalization was done with a control nontargeting siRNA placed on each individual assay plate. Q1, Q2, and Q3 values were calculated as described above. Averages and SE calculations were done for triplicates of log(x/median) values. Upper and lower boundaries were calculated as Q3 + 2c(Q3 - Q2) and Q1 - 2c(Q2 - Q1), respectively, for c = 1.7239, corresponding to a high stringency targeted error rate (α = 0.0027), and c = 0.9826, corresponding to a lower stringency targeted error rate (α = 0.046; ref. 8). High stringency hits were chosen as those targets that scored α ≤ 0.0027 in both plate-by-plate and experiment-wide analyses. Selected targets clustered within the first two plates (numbers 1-150, Fig. 1, red circles) represented hits scored as significant based on the high stringency criteria in an experiment-wide analysis only and not in a plate-by-plate analysis (listed in Supplementary Table S3). This ensued from the alphabetical clustering and nonrandom placement of siRNA sequences on the plates by the vendor, resulting in over-representation of hits in the first two plates, thereby infringing on the sparse-hit hypothesis, which assumes that hits are randomly spaced throughout the dataset.

**Results**

Kinase-targeted high-throughput siRNA screen of Wnt/β-caten- dependent transcription. We used a synthetic oligonucleotide siRNA library targeting known and predicted human kinases in a high-throughput screening format for systematic yet unbiased target discovery in contexts that were both physiologically relevant and therapeutically alluring (3, 4). HEK293 cells stably expressing a luciferase transcriptional reporter of Wnt/β-caten target gene activation (STF293 cells; ref. 9) were transfected with
1,382 siRNA duplexes targeting 691 distinct genes as a pool of 2 duplexes per target in a 96-well high-throughput screen (Supplementary Fig. S1), and subsequently treated with Wnt3a-conditioned media (Wnt3a; ref. 10) to emulate pathway activation. Human cells, together with a functionally targeted synthetic oligonucleotide library, were used to maximize the discovery of potential disease-relevant candidates and minimize off-target effects, such as those that can be observed in genome-wide screens conducted in model organisms (11, 12). A highly stringent targeted error rate in a quartile-based analysis (ref. 8; see Materials and Methods) was used to uncover candidates of statistical relevance (Fig. 1). Eleven kinases (Cdc2L1, Pank2, Lmtk3, Fli1, ErbB3, Fgr, Mapk10, Phkg1, Phkg2, Pbk, and Stk24), representing both serine/threonine and TKs, were recovered as hits, all of which were kinases not previously associated with the Wnt pathway (Supplementary Table S1). Importantly, analysis at lower stringency revealed additional kinases, several of which have known Wnt pathway regulatory functions (transforming growth factor-β receptor, NLK, CK1/2, CDKN2a, AKT2/3, and GSK3α/β), which served to authenticate the screen (Supplementary Fig. S2; Supplementary Table S2). Also, a subset of kinases enriched in cell cycle regulators emerged from an experiment-wide analysis, including the noteworthy genes Cdk8 (13), ATM, and Plk3 (Supplementary Table S3). The loss of one subset of kinases (Cdc2L1, VEGFR1/Fit1, Pank2, and Lmtk3) resulted in reduced β-catenin-dependent transcription (Fig. 1), representing potential targetable therapeutic genes.

Validation of hit genes with the use of shRNA knockdown. To validate the changes in Wnt/β-catenin signaling that were observed in the primary screen, we rescreened in STF293 cells the 11 hit genes with the use of four separate shRNA sequences corresponding to each gene of interest. Plasmids bearing shRNA were chosen for the secondary screen because of their potential for higher potency, fewer off-target effects, and stability. The shRNA sequences were selected for those that target nonoverlapping segments of the coding regions of hit genes and were assessed individually as opposed to being pooled, as was done in the primary siRNA screen. Genes were qualified as reproducible if, in at least two of three separate experiments, ≥2 shRNA sequences for a target gene increased or decreased luciferase signal above or below the median of a nontargeting negative control shRNA in concordance with the trend previously observed with siRNA (see Fig. 2, which illustrates a representative experiment). Overall, shRNA against 5 of the original 11 hit genes showed the same effect on luciferase signal as was observed in the
primary screen, and were deemed reproducible: Cdc2L1, Lmtk3, Pank2, ErbB3, and VEGFR1/FH1. Only VEGFR1/FH1 was concordant in all three independent experiments.

Characterization of VEGFR1/FH1 as a “high-confidence hit”. Direct therapeutic applications may be found for candidates whose loss-of-function by RNA interference results in attenuated Wnt/β-catenin signaling, especially in the context of oncogene-addicted colon cancer cells, in which the loss of aberrantly active Wnt/β-catenin signaling results in decreased cell survival (14). In this regard, the specific identification of VEGFR1/FH1, but not VEGFR2/KDR or VEGFR3/FH4, as a “reproducible hit” was striking. VEGFR1, a receptor TK with putative weak kinase activity, is conventionally thought to function as a negative regulator of angiogenesis (15, 16), an endothelial cell process that is actively targeted in the treatment of colorectal cancers (17). To extend the cellular link between VEGFR1 function and Wnt/β-catenin signal transduction, we further validated the effects of VEGFR1 knockdown on the β-catenin–dependent transcription observed in the primary screen. With the use of STF293 cells, a pool of four independently designed sequences against VEGFR1, as well as three of these four when tested as individual siRNA sequences, resulted in knockdown of VEGFR1 (Fig. 3A) and again attenuated β-catenin–dependent transcriptional activation (Fig. 3B and C), confirming the primary screen. No effect was observed in control HEK293-Luc cells (293Luc), a line expressing luciferase (Fig. 3B and C), confirming the primary screen. No effect was observed in control HEK293-Luc cells (293Luc), a line expressing a constitutive non–β-catenin–responsive cytomegalovirus promoter (Fig. 3D).

To independently confirm that the absence of VEGFR1 resulted in attenuated Wnt signaling at the level of transcriptional activation of Wnt/β-catenin targets, we obtained embryonic stem cells derived from a VEGFR1−/− mouse (18). Importantly, quantitative reverse transcriptase-PCR–based profiling for genes implicated in Wnt signaling was done in both wild-type and VEGFR1−/− embryonic stem cells. Embryonic stem cells lacking VEGFR1 and treated with rWnt3a showed a mere 5-fold increase in expression of T (Brachyury), a specific transcriptional target of Wnt3a (19). Importantly, quantitation of the presence of Wnt3a.

Inhibition of VEGFR1 TK activity inhibits Wnt/β-catenin–dependent transcription and is synthetic lethal in colon cancer cells. Recent data indicate that the expression of VEGFR1 in primary colon carcinoma tumor samples correlates with disease progression (20). However, the mechanistic basis of this observation has not been elucidated. Thus, to directly test the effect of abrogating VEGFR TK activity on cell survival, SW480 and KM12L4a colon cancer cells, colon cancer lines that show constitutive VEGFR TK activity on cell survival, SW480 and KM12L4a, were treated with VEGFR TK inhibitor II (ref. 22; Inh II) for 72 hours (Fig. 4A). An Inh II–induced decrease in the MTS cell viability assay was seen in these Wnt/β-catenin–addicted colon cancer cells in a concentration–dependent manner. The observed IC_{50} of 336 nmol/L (for SW480) and 120 nmol/L (for KM12L4a) matched the known IC_{50} for inhibition of VEGFR1 TK activity (180 nmol/L; ref. 22). In contrast, a 72-hour MTS assay with STF293 cells, a line that exhibits normal Wnt signaling, was unaffected by Inh II even in the presence of Wnt stimulation. Furthermore, cervical carcino-

\[ \text{VEGFR1 Synthetic Lethal to Aberrant β-Catenin Activation} \]

![Fig. 3. Validation of negative regulation of Wnt/β-catenin signaling upon loss of VEGFR1 with the use of pooled and individual siRNA duplexes targeting VEGFR1. A, gel electrophoresis after semiquantitative reverse transcriptase-PCR from STF293 cells mock-transfected (mock) or transfected with individual duplexes (D1-D4) targeting VEGFR1, a nontargeting siControl sequence (siC), or a set of four pooled ON-TARGETplus sequences against VEGFR1 (pool). Gene-specific primers were used to amplify VEGFR1 or control glyceraldehyde-3-phosphate dehydrogenase (GAPDH). Effect of (B) a set of four pooled siRNA sequences against VEGFR1 or (C) individual sequences (D1-D4) targeting VEGFR1, along with nontargeting siC as well as a firefly luciferase (PGL3)-targeting siRNA on STF293 cells stimulated with Wnt3a (gray) or control media (black). Data, mean photon flux ± SE (n = 3 per condition). D, knockdown of VEGFR1 had no effect on control 293Luc cells expressing a non–Wnt-dependent reporter, both in the absence (shown) or presence of Wnt3a.]}
Inhibition of VEGFR TK activity is synthetic lethal in β-catenin–addicted colon cancer cells, acting through downregulation of Wnt/β-catenin–dependent transcriptional activation. A, inhibition of VEGFR TK activity selectively diminishes the viability of SW480 (filled circle) and KM12L4a (filled triangle) colon cancer cells, but not Wnt3a-stimulated STF293 cells (open square), control STF293 cells (open diamond), or non–colon cancer HeLa cells (open circle). Each cell type was treated with increasing concentrations of Inh II for 72 h before cell viability analysis with the use of an MTS assay. Data, mean ± SE of fold untreated absorbance values from two independent experiments (n = 3 each) for each concentration for each cell line. B, reporter concentration response in STF293 cells stimulated with Wnt3a after pretreatment with increasing concentrations of Inh II. The bioluminescence photon flux was normalized to cell viability with the use of a resazurin dye–based fluorescence assay and plotted as fold initial, fold untreated normalized photon flux ± propagated SE (n = 3 per concentration). Inset, in HEK293T cells transfected with pTOPFLASH, Inh II strongly attenuated β-catenin–dependent transcription induced by Wnt3a (black bars) but showed a statistically significant lower effect on basal transcriptional activity in the absence of Wnt3a (gray bars). The bioluminescence photon flux was normalized to cell viability with the use of a resazurin dye–based fluorescence assay and plotted as fold initial, fold untreated normalized photon flux ± propagated SE (n = 3 per concentration), *, P < 0.05, Student's t test. Two-way ANOVA also found statistically significant effects of both Wnt treatment (P < 0.05) and drug concentration (P < 0.001). C, inhibitory effect of 48-h exposure to increasing concentrations of Inh II on constitutively active β-catenin–dependent signaling in SW480 (gray bars) and KM12L4a (black bars) colon carcinoma cells transfected with pTOPFLASH. Data, mean fold-initial, fold-untreated photon flux ± propagated SE (n = 3 per concentration). *, P < 0.05; **, P < 0.01, Student's t test.

ma–derived HeLa cells, a line that does not exhibit aberrant Wnt/β-catenin pathway activation (23), also showed no response to Inh II (Fig. 4A). These data suggested that VEGFR TK inhibition was synthetic lethal to cells with aberrant Wnt/β-catenin signaling, a process essential for colon cancer cell survival (24), but not to cells with normal Wnt/β-catenin signaling. However, further analysis of the luciferase transcriptional reporter showed that treatment with Inh II still resulted in a concentration-dependent inhibition of canonical β-catenin–dependent transcription in STF293 cells (Fig. 4B) but only in the presence of Wnt3a, as confirmed by transient transfection of HEK293T cells with pTOPFLASH, a similar β-catenin–dependent bioluminescence reporter to that used in STF293 cells (Fig. 4B, inset). Finally, in SW480 and KM12L4a colon cancer cells transiently transfected with the pTOPFLASH reporter, constitutively active β-catenin–dependent transcription was also attenuated upon treatment with Inh II (Fig. 4C). These effects were recapitulated with VEGFR TK inhibitor III, a second inhibitor targeting VEGFR TK activity (ref. 25; Supplementary Fig. S3A and B), but no effects were observed in off-target transcriptional reporter cells (293Luc) as expected (data not shown). Thus, whereas inhibition of VEGFR1 TK activity blocked Wnt-induced β-catenin–dependent transcription in normal cells as well as constitutively active β-catenin–dependent transcription in colon cancer cells, it was only in colon cancer cells that VEGFR1 TK blockade was lethal. Overall, with the caveat that the pharmacologic specificity of the tested inhibitors is not fully resolved, these data showed that by targeting VEGFR1 TK activity, the therapeutically exploitable vulnerability of Wnt/β-catenin–addicted colon carcinoma was exposed.

Wnt pathway modulation by VEGFR1 is independent of GSKβ3–dependent β-catenin stabilization. Mutations in APC or β-catenin are prevalent in colon carcinoma, resulting in constitutively stabilized β-catenin that is no longer a substrate for GSK3β (1, 2). Concordantly, in STF293 cells in which β-catenin was stabilized by pharmacologic treatment with the GSK3β inhibitor SB216763 (10 μmol/L; ref. 26), knockdown of VEGFR1 with duplexes of
siRNA yielded a similar attenuation of β-catenin–dependent transcription (Fig. 5A) as VEGFR1 knockdown in the context of Wnt3a stimulation (Fig. 3C). Thus, the effect of VEGFR1 was independent of GSK3β.

**Downstream processing.** VEGFR1 did not seem to impact the processing of total β-catenin protein levels. For example, Western blot analysis of STF293 cells showed competent stabilization of β-catenin protein upon stimulation with Wnt3a, even when effective knockdown of VEGFR1 protein was achieved with siRNA (Fig. 5B) or VEGFR TK activity was blocked (Fig. 5C). Overall, these data would suggest that therapeutic interventions directed at VEGFR TK activity may be mechanistically impacting downstream processing nodes of the Wnt/β-catenin pathway within cells, such as nuclear translocation or transcriptional activation.

We thus explored the effect of VEGFR1 on nuclear translocation of β-catenin with the use of immunofluorescence microscopy. In STF293 cells, both VEGFR1 knockdown with siRNA (Fig. 6A) or pharmacologic inhibition of VEGFR TK activity with Inh II (Fig. 6B) resulted in normal Wnt-induced β-catenin accumulation.
and nuclear translocation. These data concurred with observations in ApcMin/+ mice that showed no change in nuclear β-catenin upon treatment with the VEGFR TK inhibitor AZD2171 (27). Together, the above observations suggested that regulation of Wnt/β-catenin signaling by VEGFR1 occurred at the level of post-translational modification of β-catenin as a transcriptional coregulator and/or subsequent transcriptional activation of a selected subset of β-catenin targets.

Preliminary support for VEGFR1-linked effects on post-translational modification of β-catenin was provided by analysis of KM12L4a and SW480 colon cancer cells. Inhibition of VEGFR TK activity by treatment with Inh II, followed by immunoprecipitation and western blot analysis of β-catenin in both lines, showed a dramatic decrease in tyrosine phosphorylated β-catenin. However, β-catenin total protein levels, which are canonically governed by phospho-serine/threonine–dependent proteasomal degradation, were found to be unchanged (Fig. 6C). Thus, inhibition of VEGFR1 TK activity resulted in decreased tyrosine phosphorylation of β-catenin, thereby abrogating Wnt/β-catenin–dependent pro-proliferative transcriptional activity in colon carcinoma cells per se.

Discussion

A systematic yet unbiased screening approach in the context of activated Wnt signaling in human cancer cells has enabled the discovery of novel candidate kinase regulators of the Wnt/β-catenin pathway. Whereas the Wnt pathway has been extensively pursued with the use of model organism–based genetic screens, recent observations have found newer positive functions of two classic negative regulators of the Wnt pathway, APC (28) and GSK3β (29), highlighting the inherent epistatic bias of conventional assays (30). By interrogating Wnt signaling at the level of transcriptional activation in human STF293 cells with the use of targeted RNA interference, we hoped to identify novel candidate repressors as well as activators of the Wnt/β-catenin pathway, which could be further investigated in cell-specific contexts. Additionally, when establishing a screening strategy, there may be concern that the multitude of altered functional pathways and additional dysregulated kinases in colon cancer cell lines could confound the discovery of Wnt pathway–specific regulators. For a conventional synthetic lethal assay, we reasoned that the likelihood was high that a siRNA library screen would identify candidates that simply killed colon cancer cells through other pathways and hence would give rise to off-target effects instead of direct effects on Wnt-dependent transcription. Thus, starting with STF293 cells, we showed that one identified candidate, VEGFR1/Flt1, was a regulatory target with clinical relevance by examining Wnt/β-catenin–dependent transcription in a wide variety of contexts, including genetic-null embryonic cells from a VEGFR1−/− mouse, loss-of-function by siRNA and shRNA, VEGFR TK inhibition in Wnt-responsive cells, as well as survival of Wnt/β-catenin–addicted colon carcinoma cells.

Our data provide potential mechanistic linkages in support of prior observations. For example, VEGFR1 is expressed in various colon cancer lines that show activated Wnt signaling, including SW480 and KM12L4a cells (21). In addition, preclinical studies of a hexapeptide specifically targeting VEGFR1 (31), as well as the VEGFR TK inhibitors SU11248 (32), AZD2171 (27), and CHIR-258 (33), have shown efficacy in various models of colon cancer; however, whether the mechanism involved therapeutic “anti-angiogenesis” remains unclear (21). Furthermore, we found that VEGFR1-linked regulation of β-catenin was found to be independent of GSKβ activity and β-catenin nuclear translocation while impacting β-catenin tyrosine phosphorylation, thus providing preliminary insight into a mechanism for the antiproliferation action of VEGFR1 inhibition. It is acknowledged that the exact site of tyrosine phosphorylation remains to be mapped, and the potential differential roles of soluble VEGFR1, a splice variant (34), versus full-length VEGFR1 need to be resolved. Nonetheless, the implication that VEGFR1 links TK activity, directly or indirectly, to a node of the Wnt pathway downstream of commonly occurring oncogenic mutations tremendously enhances its potential as a therapeutic target in the context of aberrant Wnt signaling in cancer.

Our siRNA-based high-throughput screen, which uncovered an unanticipated tumor cell linkage between VEGFR1 and Wnt/β-catenin, extends the context-dependent cross-talk identified between the oncogenic Flt3 and Wnt pathways in acute myeloid leukemia (35), to VEGFR1/Flt1 and Wnt/β-catenin in colon carcinoma. Indeed, comparative genomic hybridization analysis of metastatic human colon cancer shows gain of chromosomes 1p, 8q, 13q, and 20q, including a distinct amplicon ranging from 13q11 to 13q21 (36), a region that overlaps with the exact cytotegenic location of VEGFR1. Thus, our results provide an alternative tumor cell–centric basis for the partial successes of anti-angiogenic therapy being limited to only a few cancer types, such as colorectal cancer, a Wnt/β-catenin–addicted tumor type. Future studies will be pursued to confirm this in clinical specimens in the context of targeted therapy. Potentially, a mechanistically rational strategy for synthetic lethal therapy in the treatment of colon carcinoma is selective targeted suppression of VEGFR1 TK activity in the Wnt/β-catenin–addicted tumor cells themselves.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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References

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