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The Wnt signaling pathway has tumor suppressor properties in retinoblastoma

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Abstract

Retinoblastoma is a pediatric retinal tumor caused by mutational inactivation of the tumor suppressor pRb. Additional genetic changes, as yet unidentified, are believed to be required for tumor initiation. Mutations in the Wnt signaling pathway have been implicated in the pathogenesis of many cancers. Multiple Wnt pathway genes are expressed in the retina and the pRb and Wnt pathways interact biochemically, raising the possibility that alterations in the Wnt pathway contribute to retinoblastoma. Our studies showed that Wnt signaling activation significantly decreased the viability of retinoblastoma cell lines by inducing cell cycle arrest, which was associated with upregulated p53. Furthermore, immunolocalization of the Wnt signaling mediator β-catenin in human and mouse retinoblastoma tissue indicated that canonical Wnt signaling is suppressed in tumors in vivo. These studies are consistent with the Wnt pathway acting as a tumor suppressor in retinoblastoma and suggest that loss of Wnt signaling is tumorigenic in the retina.

Keywords: Wnt; β-catenin; Retinoblastoma; pRb; p53; Cell cycle; RB1

Retinoblastoma is the most common primary ocular malignancy in children, occurring in approximately 1 in 15,000 live births [1]. Although early diagnosis and aggressive treatments have greatly improved survival, current therapies often lead to pronounced visual impairment and severe systemic complications. In many cases, surviving patients have an elevated risk for additional malignancies later in life.

Retinoblastoma tumors are caused by loss or mutational inactivation of both alleles of the retinoblastoma tumor suppressor gene (*RB1*), which encodes the pRb phosphoprotein [2]. pRb is a critical regulator of cell cycle progression at the G1/S check-point. In normal cells, hypophosphorylated pRb blocks the cell cycle by binding to and repressing E2F type transcription factors. When pRb is inactivated, E2F is released and is free to induce genes that promote DNA synthesis and cell cycle progression [3]. The pRb-E2F complex also regulates apoptosis, genome integrity, and chromosomal segregation, all of which may contribute to transformation and retinoblastoma progression. Functional inactivation of pRb and deregulation of the pRb/E2F pathway are also important steps in tumor formation outside the eye [4].

Mutations in an unknown number of additional genes or pathways, subsequent to pRb inactivation, are believed to be required for retinoblastoma formation [5]. One candidate is the Wnt signaling pathway, which is critical for controlling the initiation and progression of many types of cancers [6,7]. Wnt ligands are secreted glycoproteins that bind to the coreceptors Frizzled and LRP5/6. Canonical Wnt pathway signaling is regulated by controlling the stability of its central mediator β-catenin. In the absence of

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Wnt ligands, β-catenin levels are kept low through phosphorylation by the APC-axin-GSK3β protein complex and subsequent degradation by the proteosome. Binding of secreted Wnt ligands to their receptors induces activation of Disheveled, followed by a series of molecular events that ultimately inhibit β-catenin degradation and increase β-catenin levels. β-catenin is translocated into the nucleus where it binds to T-cell factor/lymphoid-enhancing factor (Tcf/Lef) type transcription factors and initiates transcription of Wnt target genes, including genes involved in cell growth, evasion of apoptosis, stem cell phenotype, tissue invasion and metastasis [6].

In numerous cancers, including intestinal neoplasms, breast, prostate, and lung cancers, tumorigenic mutations in APC, β-catenin, and axin lead to high β-catenin levels and constitutive Wnt activation, resulting in unregulated cellular proliferation [7,8]. For this reason, manipulating the Wnt pathway has been considered as an anti-neoplastic strategy. Blocking inappropriate activation of the Wnt pathway in various cancer cell lines downregulated progrowth genes and increased programmed cell death. For example, monoclonal antibodies against Wnt1 and Wnt2 ligands had antiproliferative effects in malignant mesothelioma [9,10].

The regulation of tumor progression by the Wnt pathway is tumor- and mutation-dependent [6] and not all tumors respond to blocking Wnt signaling. Notably, inactivating mutations in the Wnt-dependent transcription factor *LEF1* were recently identified in sebaceous tumors and were associated with reduced Wnt3a ligand-dependent Wnt signaling [11], indicating that the Wnt pathway can have tumor suppressor properties. Wnt signaling has also been proposed to reduce tumor growth in certain colorectal, prostate, and liver tumors [11–15].

Retinoblastoma tumors derive from malignant transformation of a multipotent retinal progenitor cell [16]. Both the pRb and Wnt pathways have essential functions during retinal development and may have opposing effects in controlling proliferation and differentiation of progenitor cells [17,18]. pRb regulates the cell cycle exit of retinal progenitor cells at the G1 phase and these cells continue to divide and form larger retinas in the absence of pRb [19]. In contrast, blocking Wnt signaling inhibited proliferation of progenitor cells and caused premature neuronal differentiation in the developing chick retina, and over-expression of Wnt2b inhibited neuronal differentiation and induced expression of progenitor cell markers [18]. Therefore, mutations that alter Wnt signaling in $RB1^{-/-}$ retinal progenitor cells are expected to change progenitor proliferation, potentially resulting in tumorigenesis.

The association between the pRb and Wnt pathways in tumorigenesis has not yet been explored. However, recent reports indicate functional links between the two pathways in normal tissues. In mouse epidermis, deletion of the pRb homologous proteins p107 and p130 led to increased nuclear β-catenin in basal keratinocytes, suggesting that functional pRb pathway signaling involves the Wnt pathway during hair follicle development [20]. Also, the Wnt and pRb pathways interconnect through gene regulation. This interconnection could potentially regulate oncogenesis via cyclin D1, which is a downstream target gene of Wnt signaling and a critical regulator of pRb [21]. A direct association between pRb and Wnt pathways was recently shown in non-oncogenic rat1 cells. The E2F1 transcription factor, which is regulated by pRb, induced expression of axin2 (a negative regulator of Wnt/β-catenin signaling) by binding to the axin2 promoter [22]. Elevated axin2 led to decreased Wnt signaling and reduced rat1 cell survival. Extrapolation to cancer cells suggests the possibility of cooperation between the two pathways and raises the hypothesis that aberrant Wnt signaling plays a role in retinoblastoma tumorigenesis.

The biochemical association of the pRb and Wnt pathways, the overlapping expression and function of pRb and Wnt genes in retinal progenitor cells, and the activity of both pathways in regulating cellular proliferation suggest that the Wnt pathway may be involved in the initiation or progression of retinoblastoma. To address this question, we explored the role of Wnt signaling in human retinoblastoma tissue, in a mouse model of genetically acquired retinoblastoma and in human retinoblastoma cell lines.

Materials and methods

Reagents. The human retinoblastoma cell lines Weri-RB1 and Rb355, and the mouse cell line SJmRBL8, were kindly provided by Dr. Michael Dyer (St. Jude Children's Research Hospital). SJmRBL8 cells were derived from a mouse model of retinoblastoma [23] (Laurie and Dyer, in preparation). Y79 cells were obtained from the American Type Culture Collection, Manassas, VA. Antibodies against β-catenin were from Transduction Laboratories, Chemicon and Santa Cruz. The anti-p53 antibody was from Chemicon. The Dkk1 expression vector was generously provided by Dr. Christoph Niehrs. Canonical Wnt signaling was induced by SB216763 or LiCl (Sigma), recombinant Wnt3a (rWnt3a, 50 ng/ml) (R&D Biosystems) or by incubation with conditioned media prepared from mouse L-cells (ATCC) stably expressing Wnt3a at a 1:1 ratio with normal media.

Viability Assays. Viability was quantified with the XTT assay using the WST-1 reagent (Roche). Cells in triplicate wells in 96-well plates were treated with Wnt pathway regulators or controls for 24 h and formazan release was quantified at 450 nm. Each experiment was performed at least three times on different days with three replicates within an experiment. One-way analysis of variance and Tukey post-test were used for statistical analyses.

Flow cytometry and cell cycle measurement. The cells were treated with Wnt pathway reagents and then harvested, washed in PBS, and fixed in 70% ethanol for 2 h. Cell clumps were removed using a 70 μm filter and the cells were incubated in 200 μg/ml propidium iodide with 50 μg/ml RNase A. The cellular DNA content was measured in a LSR1 three laser bench top flow cytometer (Becton–Dickinson) and 1×10^4 cells were counted for each treatment.

Wnt activity luciferase assays. Cells were co-transfected with a 4:1 ratio of the TOP-FLASH luciferase reporter plasmid (a generous gift from Dr. R. Moon, University of Washington) and a LacZ-containing plasmid. The mutated FOP-FLASH plasmid was a negative control. The cells were incubated with Wnt pathway inducers for 24 h and then lysates were collected in Reporter lysis buffer (Promega). Luciferase activity was measured in a Lumistar Galaxy luminometer (BMG Labtech) and normalized to β-galactosidase activity [24].

Immunohistochemistry. Eyes were enucleated from the $LH_{BETA}T_{AG}$ mouse model of retinoblastoma [25] or from four patients with non-familial stage 5B unilateral or bilateral retinoblastoma, fixed in 4% paraformaldehyde, incubated in sucrose (5–20%), embedded in OCT, and then frozen. The slides are blocked in non-immune serum and incubated with primary antibody overnight at 4 °C, washed, and then incubated with secondary antibody. The sections were viewed using a fluorescent microscope (Zeiss Axiovert 200) and images were captured with a digital camera (Axiocam; Zeiss). Photographic and microscopic settings were kept constant for comparisons between antibody and control staining.

Reverse transcription PCR (RT-PCR). Total RNA was isolated from Y79 cells using Trizol phenol-based extraction (Invitrogen). Total human retina RNA (Clontech) composed of RNA from pooled normal retinas from multiple individuals was used as a control. Reverse transcription was performed according to standard procedures. PCR amplification was 94 °C for 45 s, 58 °C for 45 s, and 72 °C for 60 s. The PCR primers used in this study are listed in Table 1 (Supplemental data).

Results

Wnt signaling suppresses retinoblastoma tumor growth in vitro

The human retinoblastoma cell lines Y79, RB355, and Weri-RB1 have been studied extensively to characterize cellular and molecular pathways intrinsic to the growth and survival of retinoblastoma [26]. The SJmRBL8 cell line (Laurie and Dyer, in preparation) was derived from the SJ-RBL mouse model of retinoblastoma in which deletions in three tumor suppressor genes, p53, pRb, and p107, lead to aggressive metastatic retinoblastoma tumors [23].

Y79 cells treated with Wnt3a ligand had 25% lower viability than untreated cells (Fig. 1A). Furthermore, activating Wnt signaling using the GSK3β inhibitors LiCl and SB216763 significantly reduced cellular viability to 56.5% and 71.6% of the respective control treatments. Similarly, the Wnt inducers also decreased the viability of Weri-RB1 cells by 60%, RB355 cells by 65%, and SJmRBL8 cells by 40% (Fig. 1A).

The results above show that activating the Wnt pathway at its most upstream point using a Wnt ligand, and downstream, by inhibiting GSK3β-mediated destruction of β-catenin, reduced retinoblastoma viability. Although GSK3β inhibition by SB216763 and LiCl may regulate other pathways that could potentially contribute to decreased viability, the replication of our data with the Wnt3a ligand confirms the involvement of the canonical Wnt pathway.

To further confirm the role of the Wnt pathway, the cells were co-incubated with the potent secreted Wnt antagonist Dkk1 which inhibits Wnt signaling by binding to the Wnt receptor LRP5/6 [27]. Dkk1 counteracted the Wnt-dependent viability decrease when co-incubated with LiCl and Wnt3a in each cell line, increasing the viability to the level of the untreated control (Fig. 1B, compare LiCl + Dkk1 with LiCl). Furthermore, Dkk1 treatment alone increased the survival of the cell lines by up to 27% (Fig. 1B, Y79 cells, compare Dkk1 and control treatments), suggesting that Dkk1 may increase viability by inhibiting low baseline levels of Wnt activation.

To measure induction of functional Wnt signaling, we used a transcriptional luciferase reporter assay with the TOP-FLASH plasmid, which measures Wnt signaling using the endogenous β-catenin/Tcf transcriptional complex. Y79 and Weri-RB1 cells were transfected with the Wnt signaling reporter plasmids and then treated with Wnt activators. Low basal levels of reporter activity indicated that canonical Wnt signaling is minimal in the cell line (data not shown). Incubation with Wnt3a, LiCl, and SB216763 significantly increased reporter activity by up to 10-fold in Y79 cells and 5.7-fold in Weri-RB1 cells (Fig. 2), which is within the range of Wnt activation in other cell lines ([24] and data not shown). There was no induction of luciferase activity using the mutated FOP-FLASH control plasmid (data not shown). Therefore, the Wnt pathway is downregulated in retinoblastoma cell lines but it is functionally intact and can be activated by exogenous inducers.

Western blotting of β-catenin was used to confirm Wnt signaling induction in the other cell lines due to low transfection efficiency of the reporter plasmid. Elevated β-catenin, the central mediator of canonical Wnt signaling, is a well-established marker of Wnt pathway activation.

Untreated Y79 cells had low basal levels of β-catenin in whole cell lysates (UT in Fig. 2C). Treatment with Wnt3a ligand (rWnt3a and Wnt3a CM), as well as the GSK3β inhibitors LiCl and SB216763, increased β-catenin on average twofold over control incubations. Although the fold change was not as high as the luciferase reporter assay (Fig. 2), this experiment confirms that increased total β-catenin by Western blotting accurately reflects upregulated Wnt signaling. Similarly, low basal levels of β-catenin that were increased by Wnt inducers were observed in Weri-RB1, RB355, and SJmRBL8 cells (Fig. 2C). Therefore, despite having different primary mutations, canonical Wnt signaling is maintained at low levels in the four cell lines but it can be activated.

Wnt signaling induces cell cycle arrest

To examine how Wnt signaling reduces retinoblastoma viability, we determined whether Wnt activation alters progression through the cell cycle. The Y79 retinoblastoma cell line was incubated with LiCl or Wnt3a and the percent of cells in each phase of the cell cycle was measured using flow cytometry. Incubation with 40 mM LiCl resulted in the accumulation of more cells in G2/M phase and less cells in G1 (Fig. 3A). Similarly, Wnt3a treatment resulted in a larger proportion of Y79 cells in G2 phase compared with the control (Fig. 3B). Incubation of the RB355, SJmRBL8, and Weri-RB1 cell lines with Wnt signaling activators also resulted in cell cycle blocks. RB355 had more cells in G2/M phase and fewer cells in G1 when treated with LiCl, but was not affected by Wnt3a treatment at this time-point. SJmRBL8 had more cells in G1, fewer cells in S, and fewer cells in G2 when treated with LiCl, but the cell cycle was also not affected by Wnt3a. Weri-RB1 had more cells in

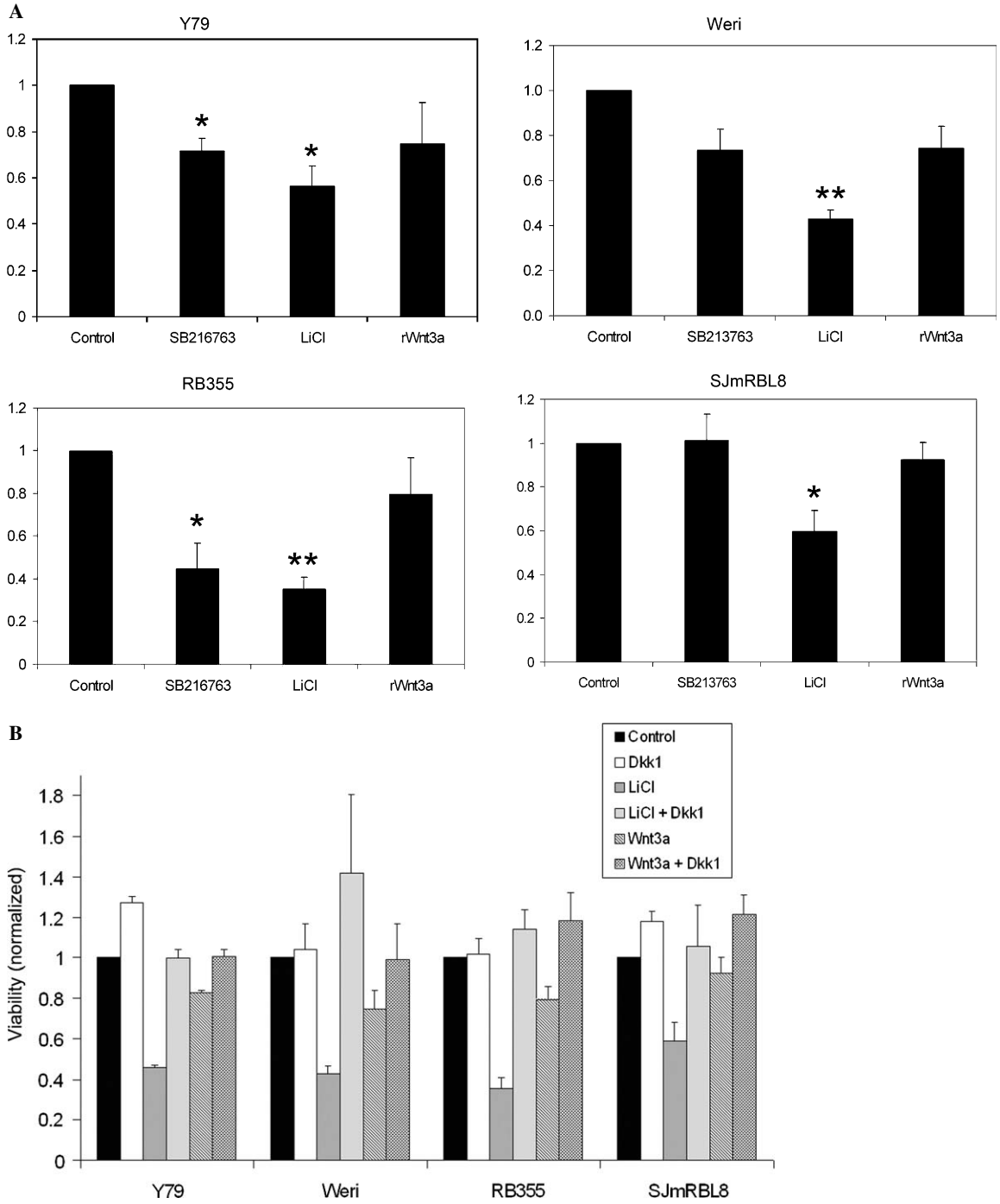


Fig. 1. Wnt signaling activators reduce retinoblastoma viability. (A) The viability of the retinoblastoma cell lines treated with Wnt signaling activators was measured using the XTT assay (mean ± SD). There was significantly lower viability when Wnt signaling was activated compared with untreated cells. Each treatment is shown as a ratio of their respective controls (DMSO for SB216763; untreated for LiCl and rWnt3a). $^{*}p < 0.01$; $^{**}p < 0.001$. (B) The secreted Wnt inhibitor Dkk1 reversed the effect of Wnt pathway activation.

S phase and fewer cells in G2 when treated with LiCl, and more cells in S phase when treated with Wnt3a (Fig. 3B). Although cells that lack normal pRb may enter S phase and undergo apoptosis or G2 arrest [28], we did not observe growth arrest until Wnt activators were added. These results indicate a Wnt-dependent block at G1, S or G2 phases in the cell lines.

To determine the extent of cell cycle arrest, we performed a time-course analysis. Y79 cells were incubated with 40 mM LiCl and flow cytometry was performed on cells collected after 24, 48, and 72 h. Y79 cells remained in G2 phase over the course of the study, consistent with a cell cycle arrest rather than a change in population doubling time (Suppl Fig 1). Similarly, at

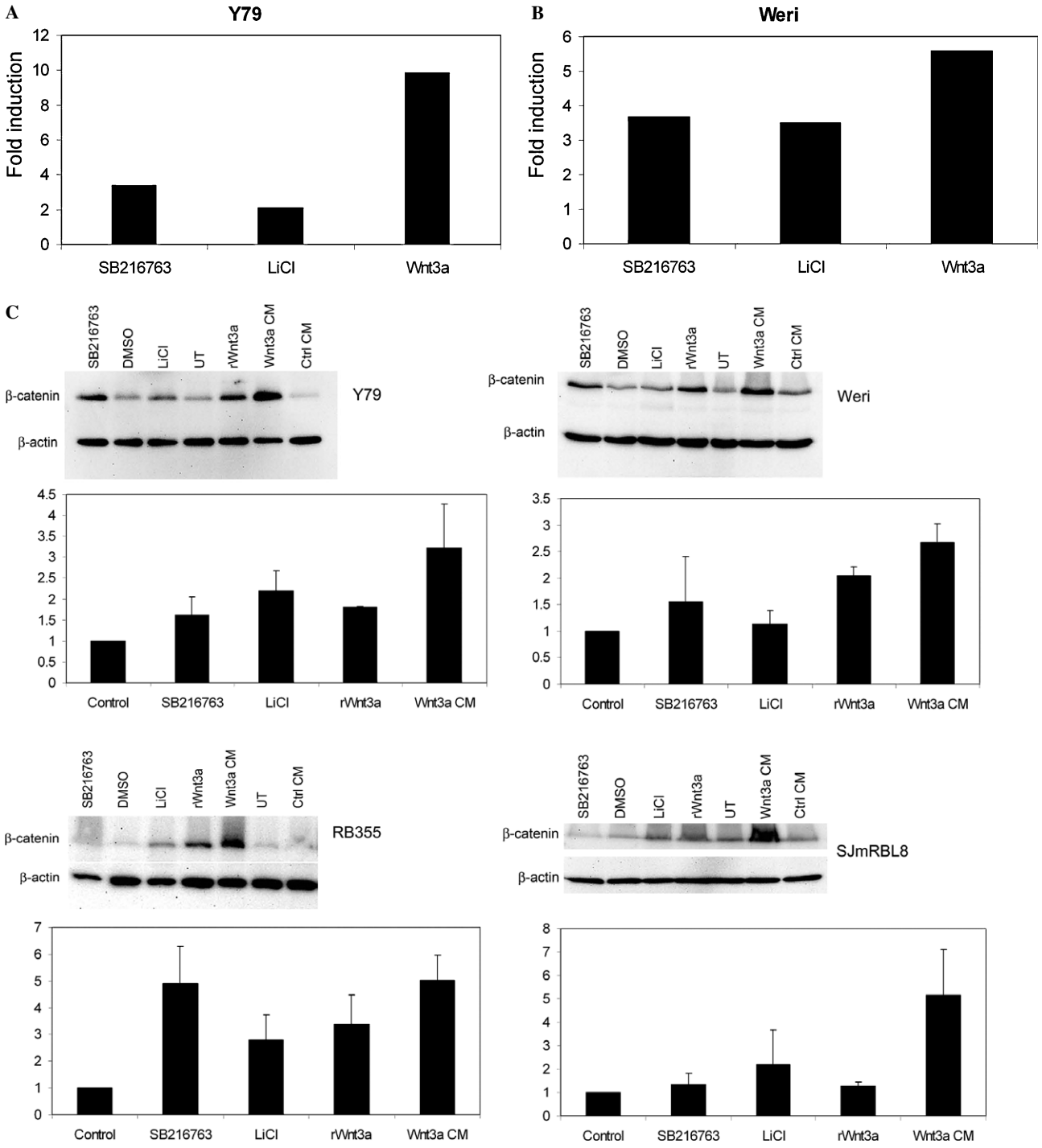
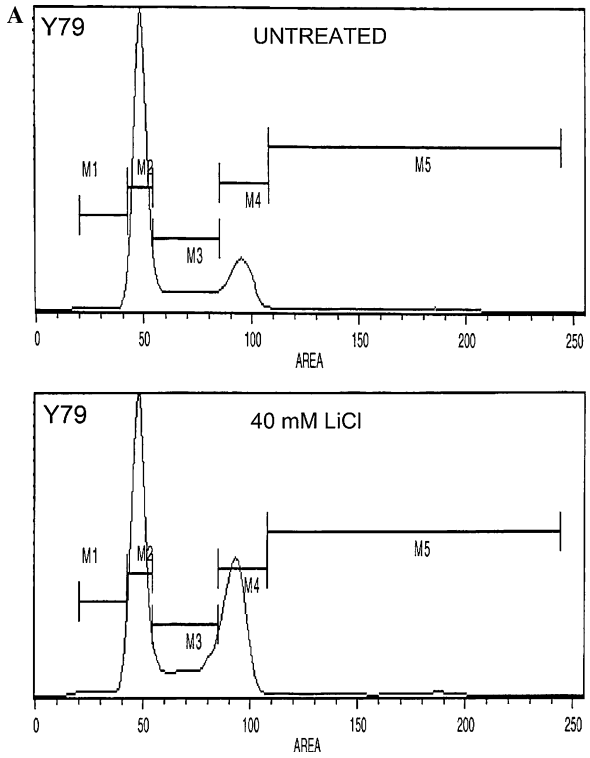


Fig. 2. Wnt signaling is activated in retinoblastoma cells. (A) The Wnt pathway was induced in Y79 cells by the Wnt3a ligand or by the GSKβ inhibitors LiCl and SB216763, and Wnt signaling was measured by the luciferase reporter assay. β-Galactosidase activity was used for normalization. Fold induction is shown relative to the control treatments. (B) The Wnt pathway was also induced in Weri-RB1 cells. (C) Baseline levels of β-catenin are low but incubation with the Wnt activators SB216763, LiCl, Wnt3a ligand-containing conditioned media (CM), and purified recombinant Wnt3a ligand (rWnt3a) stabilized and increased β-catenin, indicating activation of the canonical Wnt pathway. β-Actin was used for normalization. A representative Western blot is shown and β-catenin/β-actin levels from four independent experiments were quantified.

48 h, Weri-RB1 had 98% more cells in S phase when treated with LiCl and 17% more cells in S phase when treated with Wnt3a (data not shown). SJmRBL8 cells also retained the cell cycle block, showing 57% more cells in G1 and 72% fewer cells in G2 at 48 h of treatment (data not shown). The RB355 cells did not retain substantial differences in the cell cycle at 48 h of treatment. Therefore, Wnt signaling is anti-proliferative in retinoblastoma cells, in contrast to its growth promoting effect in other tumor cells.



B

Y79

	untreated	LiCl	control	Wnt3a
G1	61.46	46.00	52.99	50.16
S	17.88	18.89	14.21	12.45
G2/M	19.71	33.75	33.21	37.85

RB355

	untreated	LiCl	control	Wnt3a
G1	38.34	33.63	38.36	39.96
S	44.66	42.65	44.69	44.06
G2/M	17.97	25.16	18.01	17.83

SJmRBL8

	untreated	LiCl	control	Wnt3a
G1	42.27	25.87	38.76	41.55
S	40.07	47.38	39.96	39.34
G2/M	18.83	27.47	22.09	19.78

Weri

	untreated	LiCl	control	Wnt3a
G1	37.00	27.26	40.65	37.56
S	22.02	46.27	25.24	28.72
G2/M	35.88	25.87	31.30	30.96

Fig. 3. Wnt signaling activation leads to cell cycle arrest. (A) Wnt signaling was activated in Y79 cells by 40 mM LiCl and the cell cycle was quantified using flow cytometry. A G2 phase arrest is indicated by the larger M4 peak compared with untreated cells (M1, subG1 phase; M2, G1 phase; M3, S phase; M4, G2/M phase; M5, polyploid). Representative example from four trials is shown ($p < 0.01$). (B) The percent of cells in each phase of the cell cycle are shown for cells incubated with 40 mM LiCl and 50 ng/ml recombinant Wnt3a, compared with controls, demonstrating that Wnt pathway activation by GSK3β inhibition or ligand binding leads to cell cycle arrest in the retinoblastoma cell lines.

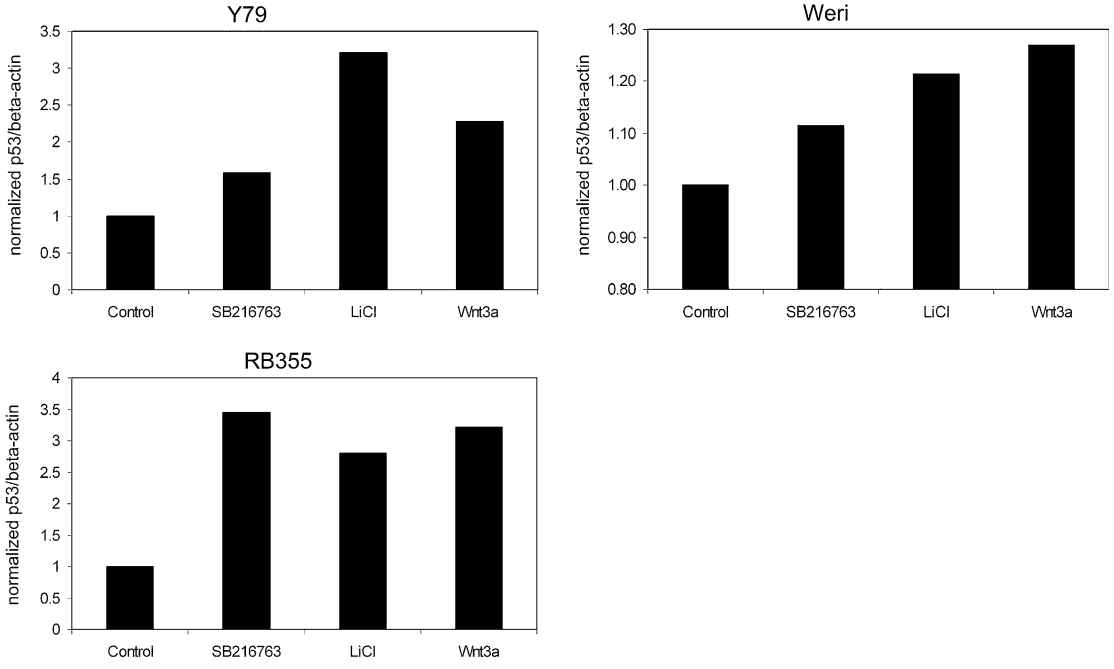


Fig. 4. p53 expression is increased by Wnt signaling activation. Incubation of Y79, Weri-RB1, and RB355 cells with the Wnt activators SB216763, LiCl and recombinant Wnt3a ligand is associated with increased p53 levels. p53/β-actin levels from three independent experiments were quantified.

The tumor suppressor 53 is rarely mutated in human retinoblastoma, although recent evidence suggests that it may be functionally inactivated [29]. p53 mediates the cellular response to stress by controlling apoptosis and regulating cell cycle arrest at G1 and G2 phases. Wnt signaling regulates p53 expression, increasing p53 levels in some cell types

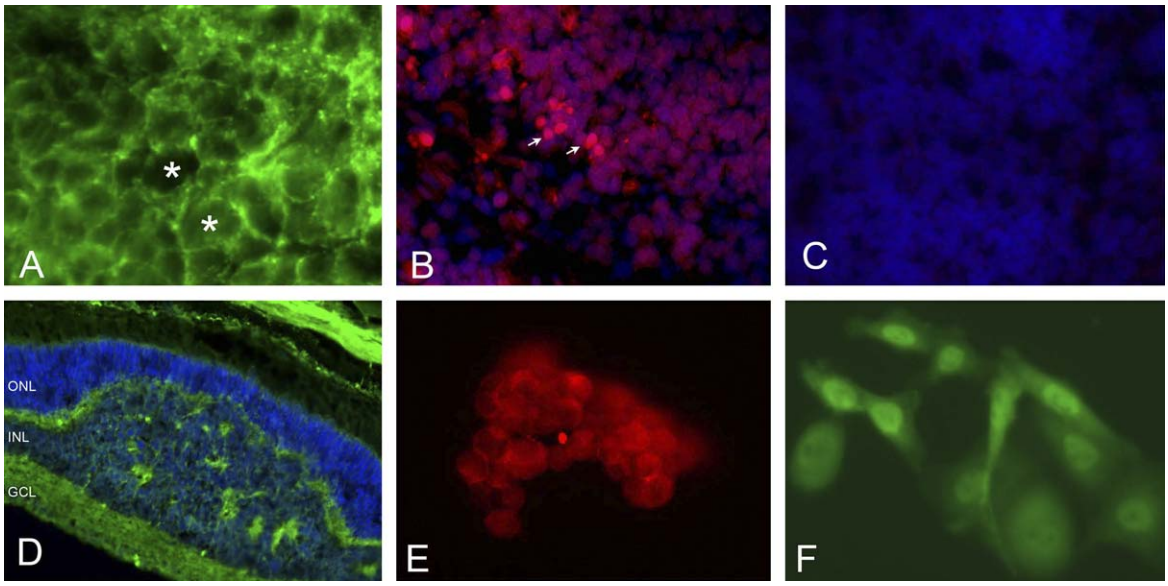


Fig. 5. Wnt signaling is not substantially activated in retinoblastoma tumors. (A) Immunodetection of nuclear β-catenin was used as a cellular marker for activated canonical Wnt signaling. β-Catenin (green) is localized primarily to the membrane or cytoplasm of retinoblastoma tumor cells and not in the nuclei (*) (400×). (B) In one out of the four tumors tested, in this case immunostained with a second β-catenin antibody (red), several cells had nuclear localized Wnt signaling (arrows) (630×). (C) The negative control, incubated without the primary antibody, demonstrated antibody specificity (630×). (D) A medium-sized tumor from an $LH_{BETA}T_{AG}$ mouse, a murine model of retinoblastoma, also does not have nuclear β-catenin staining (400×). β-Catenin is localized to the cytoplasm and synaptic layers. (E) The retinoblastoma cell line Y79 does not have nuclear β-catenin, indicating that Wnt signaling is also not activated in these cells. (F) The colorectal carcinoma cell line SW480 has nuclear and cytoplasmic β-catenin, indicating activated Wnt signaling. ONL, photoreceptor outer nuclear layer; INL, inner nuclear layer; GCL, ganglion cell layer.

and decreasing p53 in other cells [30]. We found that in Y79, Weri-RB1, and RB355 cells, Wnt pathway activation upregulated p53 levels up to 3.4-fold (Fig. 4). p53 is deleted in SJmRBL8 and was not examined. These results suggest that p53 may be involved in Wnt-dependent changes in cell cycle progression.

Wnt signaling is not activated in retinoblastoma tumors

The Wnt-dependent cell cycle arrest and the low β-catenin levels in the retinoblastoma cell lines raise the possibility that Wnt signaling may be suppressed in retinoblastoma tumors in vivo. To explore Wnt pathway activation in retinoblastoma, nuclear localization of β-catenin was used as a marker.

Analysis of four human retinoblastoma tumors demonstrated that β-catenin was predominantly membrane-associated and appeared in an irregular honeycomb pattern (Fig. 5A). Similar results were observed using two additional anti-β-catenin antibodies (Fig. 5B and data not shown). In one out of the four tumors a small number of cells had nuclear localized β-catenin (Fig. 5B, arrows), which may represent a different cell type within the tumor or could be retinoblastoma cells that are phenotypically different from the rest of the tumor. This nuclear staining also provides an internal positive control and demonstrates that lack of nuclear localization in the majority of this tumor and in the other tumors cannot be attributed to inappropriate tissue processing. The immunodetection specificity was demonstrated by lack of staining in sections that were incubated with normal serum instead of the primary antibody (Fig. 5C). We also demonstrated that Y79 cells did not have nuclear β-catenin (Fig. 5E), as expected from the luciferase and Western blotting results above. In contrast, the colorectal cancer cell line SW480, which has constitutive Wnt/β-catenin signaling, had intense nuclear β-catenin staining (Fig. 5F).

The above data demonstrate that retinoblastoma does not have predominantly nuclear localized β-catenin, indicating that the Wnt pathway is not active in retinoblastoma. In this regard, retinoblastoma differs from most other tumor types, which have elevated Wnt signaling as an important feature of their pathogenesis [6,7]. Although four tumors are insufficient for statistical analyses, the lack of Wnt activation in the tumors in combination with the Wnt-dependent repression of the cell cycle and viability is consistent with a tumor suppressor role for the Wnt pathway in retinoblastoma.

To characterize Wnt signaling activation during tumor progression, we used the $LH_{BETA}T_{AG}$ mouse model of retinoblastoma [25]. Functional inactivation of pRb occurs in the $LH_{BETA}T_{AG}$ mice by over-expression of SV40-large T antigen, which binds to and alters the activity of p53, pRb and the retinoblastoma family members p107 and p130. The retinal tumors in $LH_{BETA}T_{AG}$ mice mimic the histopathology of human retinoblastoma, including the formation of stereotypic rosettes, local invasion, extensive vascularization and similar antigenic reactivity as their

human counterpart. These mice have been used extensively for investigating pathways involved in the progression of retinoblastoma and the response to therapy.

We analyzed mice that had small, medium, and large sized tumors. In the adjacent non-tumor tissue, β-catenin was localized to the inner and outer plexiform layers and the nerve fiber layer (Fig. 5D). This pattern of staining is consistent with previous reports [31]. Within the tumor, β-catenin was exclusively cytoplasmic or membrane-associated. The staining intensity was heterogeneous, with some regions showing very intense staining. We did not detect nuclear β-catenin in the mouse tumors regardless of tumor size or antibody used in the five mice tested (Fig. 5D). These results indicate that the Wnt pathway is not active in retinoblastoma tumors in the $LH_{BETA}T_{AG}$ mouse model, similar to the human tumor samples.

To characterize which Wnt pathway genes are expressed in retinoblastoma tumor cells, we performed PCR on cDNA derived from Y79 cells (Suppl Table 1). Y79 cells express multiple Wnt pathway genes (Suppl Fig. 2). The central mediators of canonical Wnt signaling, GSK3β and β-catenin, were expressed, as were Wnt pathway inhibitors that are frequently absent in other cancers, including APC, axin1, and axin2. Interestingly, several retinal genes that regulate the Wnt pathway, such as Dkk3, Wif-1, Wnt2, and Wnt3, were not expressed in the Y79 cells. These gene expression changes may contribute to downregulated Wnt signaling in retinoblastoma.

Discussion

The Wnt pathway is a critical regulator of tumor initiation and progression in many malignancies. Wnt signaling is elevated in numerous cancer types, including colorectal carcinoma, breast, prostate, and lung cancers [6,7]. In contrast, we found that Wnt signaling is not active in human and mouse retinoblastoma tissue and cell lines, indicating that constitutive Wnt signaling is not required for retinoblastoma tumorigenesis. Furthermore, Wnt signaling decreased the proliferation of retinoblastoma cells by inhibiting cell cycle progression. These data indicate that the Wnt pathway is a tumor suppressor in retinoblastoma.

Our data suggest that inappropriate silencing of the Wnt pathway may promote tumorigenesis in the retina and further imply that Wnt inactivating mutations may be present in retinoblastoma. Inactivating mutations were recently identified in the Wnt-dependent transcription factor *LEF1* in sebaceous tumors and tumorigenesis was associated with reduced Wnt signaling [11]. A tumor suppressor role for Wnt signaling was also suggested in several other tumors. For example, Wnt pathway activators induced p53-dependent apoptosis and p53-independent cell cycle arrest in colorectal cancer cells [12] and reduced the growth of androgen receptor-positive prostate cancer cell lines [13]. Furthermore, Wnt activation was also associated with decreased proliferation and G1/S arrest in nine out of 12 hepatocellular carcinoma cell lines [14]. The lack of nuclear β-catenin in other tumor types, such as pancreatic tumors [32] and malignant ovarian germ cell tumors [33], provides a compelling rationale to explore an anti-neoplastic role for Wnt in those cancers.

In many cell types p53 controls apoptosis and regulates cell cycle arrest at G1 and G2 [34,35]. Retinoblastoma cells treated with Wnt signaling activators upregulated p53, confirming Wnt-dependent p53 induction observed in other cells [36]. Wnt signaling regulates p53 expression, by increasing p53 levels in some cell types and decreasing p53 in others [30]. p53 also regulates Wnt signaling and the interaction between p53 and GSK3β links the Wnt pathway with the Akt/PKB cell survival pathway. The balance between Wnt signaling, p53, and Akt may mediate whether a stimulated cell undergoes apoptosis, cell cycle arrest or transformation.

Although pRb mutations cause p53-dependent apoptosis, most retinoblastoma tumors contain wild-type p53, despite the fact that p53 loss would confer a survival advantage [37]. However, p53 may be functionally inactivated in retinoblastoma due to increased p53 inhibitor proteins [29]. This idea is supported by the fact that two commonly used mouse models of retinoblastoma require functional or genetic inactivation of p53 to form retinoblastoma-like tumors [23,25]. Interestingly, p53 and p21 expression varies with the differentiation state within human retinoblastoma tumors, suggesting that p53 may promote cell death or cell cycle arrest at different stages of retinoblastoma development [37]. Future studies will address whether p53 mediates Wnt-dependent cell cycle arrest in retinoblastoma. Exploring the role of p53 in the Wnt response in retinoblastoma may also provide potential targets for therapy.

In the developing retina, pRb regulates the cell cycle exit of retinal progenitor cells at G1 phase [19]. Our findings suggest that Wnt signaling may normally act as a safety mechanism to halt proliferation in $pRb^{-/-}$ cells that have escaped the pRb-regulated G1 checkpoint. Because retinoblastoma tumorigenesis requires the accumulation of additional gene defects in $pRb^{-/-}$ cells [5], secondary mutations that decrease Wnt signaling would cause the cells to bypass a cell cycle block, permitting increased proliferation.

Our results suggest that activating the Wnt pathway should be tested for its anti-neoplastic potential in mouse models of retinoblastoma. Additionally, mutations in *RB1* are found in most cancers and inactivation of pRb is an important event in tumor progression [4]. Therefore, characterizing the interaction of the Wnt and pRb pathways will lead to insights into the control of dysregulated proliferation in many cancer types.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bbrc.2006.08.044.

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