

Activated Wnt/ β -catenin signaling in melanoma is associated with decreased proliferation in patient tumors and a murine melanoma model

Andy J. Chien^{a,b,1}, Erin C. Moore^{b,c,1}, Anke S. Lonsdorf^d, Rima M. Kulikauskas^{a,b}, Bonnie Gould Rothberg^e, Aaron J. Berger^e, Michael B. Major^{a,c}, Sam T. Hwang^d, David L. Rimm^e, and Randall T. Moon^{a,c,f,2}

^aInstitute for Stem Cell and Regenerative Medicine, ^bDepartment of Medicine, Division of Dermatology, and ^dDepartment of Pharmacology, University of Washington School of Medicine, Seattle, WA 98195; ^cThe Howard Hughes Medical Institute and ^eDermatology Branch, Center for Cancer Research, National Cancer Institute, Bethesda, MD 20815; and ^fDepartment of Pathology, Yale University, New Haven, CT 06520

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This study demonstrates that in malignant melanoma, elevated levels of nuclear β -catenin in both primary tumors and metastases correlate with reduced expression of a marker of proliferation and with improved survival, in contrast to colorectal cancer. The reduction in proliferation observed in vivo is recapitulated in B16 murine melanoma cells and in human melanoma cell lines cultured in vitro with either WNT3A or small-molecule activators of β -catenin signaling. Consistent with these results, B16 melanoma cells expressing WNT3A also exhibit decreased tumor size and decreased metastasis when implanted into mice. Genome-wide transcriptional profiling reveals that WNT3A up-regulates genes implicated in melanocyte differentiation, several of which are down-regulated with melanoma progression. These findings suggest that WNT3A can mediate transcriptional changes in melanoma cells in a manner reminiscent of the known role of Wnt/ β -catenin signaling in normal melanocyte development, thereby altering melanoma cell fate to one that may be less proliferative and potentially less aggressive. Our results may explain the observed loss of nuclear β -catenin with melanoma progression in human tumors, which could reflect a dysregulation of cellular differentiation through a loss of homeostatic Wnt/ β -catenin signaling.

differentiation | prognosis | metastasis | WNT5A | B16 model | microarray

Malignant melanoma accounts for <5% of all skin cancers, yet is responsible for 80% of skin cancer deaths (1). The outlook for patients with metastatic melanoma remains quite bleak, with a 5-year survival rate of only 5%–15% that has not changed significantly over decades despite intensive efforts to develop an effective therapy. Although the molecular mechanisms underlying the formation and progression of melanoma remain unresolved, recent studies have implicated Wnt signal transduction pathways in melanoma biology (2), raising the question of whether this insight can be used to develop a therapy.

Wnt genes encode a family of 19 secreted glycoproteins that act as ligands to activate receptor-mediated signaling pathways that control cell fate and differentiation, cell proliferation, and cell motility (3). The extensively characterized Wnt/ β -catenin pathway inhibits the degradation of β -catenin, leading to its accumulation in the nucleus and to regulation of target gene expression (3). Vertebrates also have at least one other Wnt signaling pathway, often referred to as “noncanonical” Wnt signaling, that uses β -catenin-independent signaling mechanisms and, in some contexts, actively antagonizes β -catenin signaling (3–5). Both Wnt pathways have been implicated in cancer. Specifically, mutations leading to constitutive activation of Wnt/ β -catenin signaling are observed in colorectal cancer and in some kidney tumors (6–9), where activation of the pathway has been directly implicated in disease pathogenesis. This finding, coupled with the initial identification of vertebrate *Wnt1* as an oncogene in a breast cancer screen (10) and with studies demonstrating the

activation of this pathway in other cancers, has promoted the idea that elevated Wnt/ β -catenin signaling is oncogenic in most contexts (3). In colorectal carcinoma, the majority of tumors exhibit constitutive activation of Wnt/ β -catenin signaling through activating mutations of the adenomatous polyposis coli (*APC*) gene, and the presence of increased nuclear β -catenin has been shown to predict cancer progression to metastasis, as well as decreased patient survival (11–14). Interestingly, although activating mutations in the β -catenin pathway are rare in melanoma (15–20), the noncanonical pathway, often activated by WNT5A, has been implicated in melanoma metastasis (21–24).

Melanocytes arise from neural crest cells, a multipotent pool of precursors that also give rise to neuronal, glial, and cartilage lineages (25). Wnt/ β -catenin signaling is necessary and sufficient to drive neural crest cells toward a melanocyte cell fate, in large part through direct regulation of transcriptional targets, such as the homeobox gene *microphthalmia transcription factor (MITF)* (26–28). The observed presence of nuclear β -catenin in the majority of benign nevi, along with the loss of nuclear β -catenin seen with melanoma progression (29–31), support the hypothesis that activation of Wnt/ β -catenin signaling is important for cellular homeostasis in this context. Consequently, the dysregulation of specific transcriptional programs in melanocytes and nevus cells through a loss of Wnt/ β -catenin signaling may contribute to the lower survival seen in patients with tumors that lack nuclear β -catenin (29–31).

In the present study, we found that elevated levels of nuclear β -catenin correlate with improved survival from melanoma. This finding is paralleled in an established murine melanoma model in which activation of Wnt/ β -catenin by either WNT3A or small molecules leads to decreased proliferation in vitro and decreased tumor size in vivo. We also noted that activation of Wnt/ β -catenin signaling promotes the expression of markers of melanocyte differentiation, which correlates with our observed decrease in the expression of proliferation markers in patients with higher levels of nuclear β -catenin. Interestingly, the same genes activated by Wnt/ β -catenin signaling are antagonized by WNT5A, which may be relevant to disease progression given the

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¹A.J.C. and E.C.M. contributed equally to this work.

²To whom correspondence should be addressed. E-mail: rtmoon@u.washington.edu.

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observed increased expression of *WNT5A* in later-stage, more aggressive melanomas (21–24). Together, these findings support the hypothesis that melanoma progression is associated with a loss of Wnt/ β -catenin signaling, leading to dysregulated cell fate and increased proliferation.

Results

Nuclear β -Catenin Levels Correlate with Improved Patient Survival. In a tissue microarray composed of 343 melanoma tumor cores (118 primary tumors plus 225 recurrences/metastases), we used immunohistochemical staining followed by automated quantitative analysis (AQUA[®]) to measure nuclear β -catenin levels. Fig. 1A shows representative immunofluorescent staining from 2 different tumor cores measured by AQUA (32). The tumor mask was defined by S100 staining, and nuclei were defined by labeling with DAPI. This method provides a clear distinction between nuclear and cytoplasmic/membranous β -catenin. Nuclear β -catenin is higher in primary tumors than in metastases (bordering on statistical significance by nonparametric *t*-test), suggesting that melanoma progression is associated with a loss of Wnt/ β -catenin signaling (supporting information (SI) Fig. S1C).

Survival probabilities were derived using Kaplan-Meier analysis after primary tumors were stratified into tertiles based on nuclear β -catenin expression (Fig. S1A), with the upper tertile corresponding to tumors in the highest third of nuclear β -catenin expression and the lower tertile corresponding to tumors in the lowest third of nuclear β -catenin expression. By using these AQUA-scored tumor samples to correlate different levels of nuclear β -catenin with survival, we found that higher expression of nuclear β -catenin in primary tumors predicts increased survival (Fig. 1B). In addition, metastases and recurrences with the highest nuclear β -catenin levels (Fig. S1B) also are associated with a higher survival probability (Fig. 1C). These findings provide an initial survival analysis in metastatic melanoma based on nuclear β -catenin levels and, more importantly, suggest that even in advanced disease, activation of Wnt/ β -catenin signaling still provides a survival benefit. A detailed comparison of these results in the context of previously published analyses of nuclear β -catenin levels in melanoma is presented in *SI Discussion*.

Because we had tumor depth measurements (Breslow thickness) for 113 primary tumors in our array cohort, we analyzed this subgroup of patients based on the Breslow thickness stratification used in the 2002 American Joint Committee on Cancer (AJCC) melanoma staging criteria (33). Survival analysis correlates well with AJCC tumor staging by Breslow thickness (Fig. S1D). We found a significant decrease in nuclear β -catenin levels with increasing tumor thickness (Fig. 2A). In contrast, the tumor proliferation index, as measured by the Ki-67 marker, increased with more advanced tumor staging (Fig. 2B).

Strikingly, distribution histograms of %Ki-67 staining in primary tumors stratified by nuclear β -catenin expression showed a statistically significant shift toward increased proliferation (elevated %Ki-67 staining) in the groups with lower nuclear β -catenin levels (Fig. 2C). No significant difference in the distribution of α -catenin was seen within these tertiles (Fig. S2A). Ki-67 exhibited similar results within tumors to another proliferative marker, PCNA (Fig. S2C). A Deming regression of nuclear β -catenin and %Ki-67 within primary tumors is shown in Fig. 2D (slope = -1.089 ± 0.2374 ; $P < .0001$). In contrast, no relationship was found between α -catenin expression and %Ki-67 staining (Fig. S2B). Together, our data from patient tumors support a model in which activation of Wnt/ β -catenin signaling is associated with decreased proliferation.

Activation of Wnt/ β -Catenin Signaling Is Correlated with Decreased Proliferation of Melanoma Cells. We next investigated whether Wnts elicit changes in melanoma cells cultured in vitro that might be consistent with our clinical observations. Because

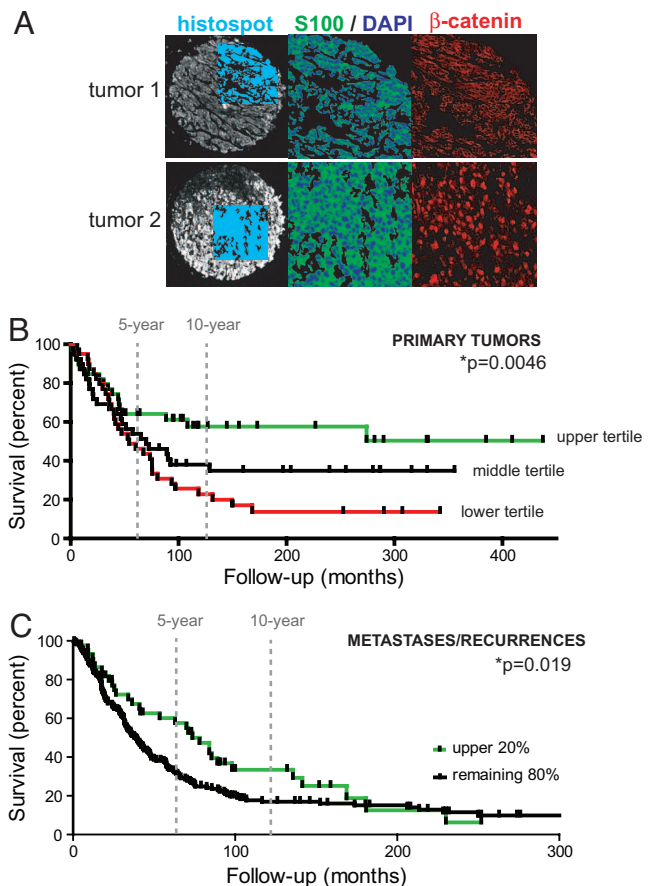


Fig. 1. Nuclear β -catenin levels predict improved survival in melanoma patients. (A) Representative tumors cores from the tissue microarray are shown to illustrate the localization of nuclear β -catenin by AQUA. Tumor 1 (Upper panels) is representative of tumors with lower nuclear β -catenin expression, whereas tumor 2 (Lower panels) is representative of tumors with higher nuclear β -catenin expression. In the left panels, the orientation of the histospot used for analysis is oriented on the tumor core. The middle panels illustrate how S100 and DAPI are used to identify the cytoplasmic/membranous and nuclear compartments of the tumor, respectively. Staining with β -catenin, shown in the right panels, is co-localized with either S100 or DAPI to generate measured values of β -catenin staining in each subcellular compartment. (B) Primary tumors ($n = 118$) were stratified a priori into tertiles based on nuclear β -catenin level (see also Fig. S1A). Patients with the highest nuclear β -catenin levels (upper tertile) have a significantly higher survival probability by Kaplan-Meier analysis compared with those in the middle and lower tertiles (log-rank test). (C) Metastatic and recurrent tumors were separated into those with the highest nuclear β -catenin levels (upper 20%; $n = 46$) and those with lower nuclear β -catenin levels (remaining 80%; $n = 179$). The nuclear β -catenin levels in the upper 20% of metastatic/recurrent tumors correspond with the levels seen in the upper tertile of primary tumors (Fig. S1B). Kaplan-Meier analysis demonstrated a significantly increased survival probability in patients with the highest nuclear β -catenin levels (Gehan-Breslow-Wilcoxon test).

melanoma tumors express *WNT3A* (Fig. S3A), which plays a pivotal role in the regulation of melanocyte biology (28, 34), as well as *WNT5A*, which is elevated in melanoma metastases (21–24), we transduced B16-F1 mouse melanoma cells with lentivirus constructs encoding *WNT3A*, *WNT5A*, or a *GFP* control. We designate the resulting cell lines B16:*GFP*, B16:*WNT3A*, and B16:*WNT5A*, respectively.

Scoring cells for nuclear accumulation of β -catenin revealed that only B16:*WNT3A* cells, not B16:*WNT5A* or B16:*GFP* cells, exhibit elevated β -catenin (Fig. 3A). Expression of *WNT5A* was confirmed by immunoblot analysis (data not shown). As a positive control, we

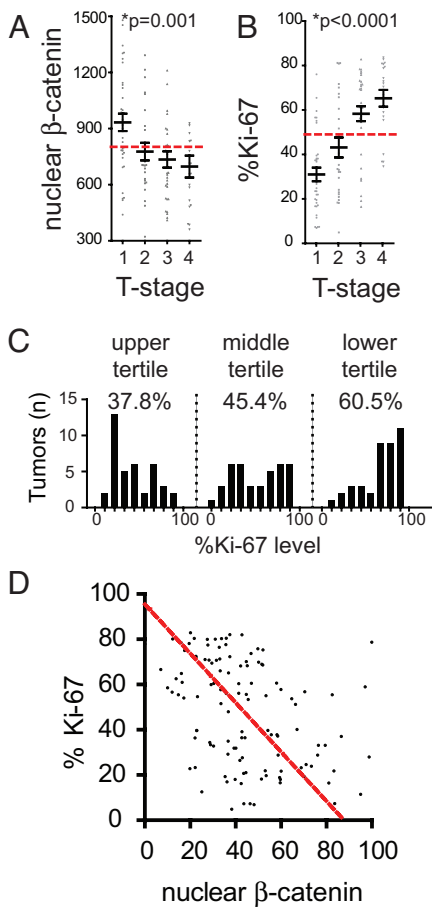


Fig. 2. Activated Wnt/β-catenin signaling is associated with decreased proliferation. (A) Nuclear β-catenin level was measured in primary tumors staged by Breslow depth according to AJCC criteria. Tumor depth increases from T1 to T4. Bars representing the mean and SEM for tumors in each stage indicate decreasing nuclear β-catenin level with increasing tumor depth. (B) In comparison, the proliferative index of tumors, as measured by %Ki-67, increases with tumor depth. Gray dots represent individual tumors, and the dotted red line indicates the average for the entire cohort. The changes in nuclear β-catenin and Ki-67 were highly significant by ANOVA followed by a posttest for linear trend. (C) Histograms binned by 10% increments reveal the distribution of %Ki-67 within tumors stratified into tertiles by nuclear β-catenin level. Note the increased number of tumors with higher %Ki-67 in the presence of lower nuclear β-catenin levels (lower tertile), compared with the larger number of tumors with lower %Ki-67 seen in the presence of higher nuclear β-catenin levels (upper tertile). The mean %Ki-67 for each tertile (shown above the histograms) increased significantly with lower expression of nuclear β-catenin ($*P < 0.0001$ by ANOVA with posttest for linear trend). (D) Levels of nuclear β-catenin and %Ki-67 in individual tumors were analyzed by Deming regression, revealing a slope of -1.089 ± 0.2374 , suggesting that higher levels of nuclear β-catenin are associated with decreased %Ki-67. In contrast, Deming regression comparing α-catenin and %Ki-67 revealed a slope not significantly different from zero (Fig. S2B).

noted that conditioned media (CM) from B16:WNT3A cells, but not from B16:WNT5A cells or B16:GFP cells, activate a β-catenin-responsive reporter in UACC1273 melanoma cells (Fig. 3B), confirming that B16:WNT3A cells secrete active WNT3A. We then found that B16:WNT3A cells exhibit marked up-regulation of the β-catenin target gene *Axin2* (35) compared with B16:GFP and B16:WNT5A cells (Fig. 4).

In vitro cell proliferation studies using the MTT cell proliferation assay or manual cell counts showed that B16:WNT3A cells exhibit significantly decreased proliferation compared with B16:GFP or B16:WNT5A cells (Fig. 3C). This finding was

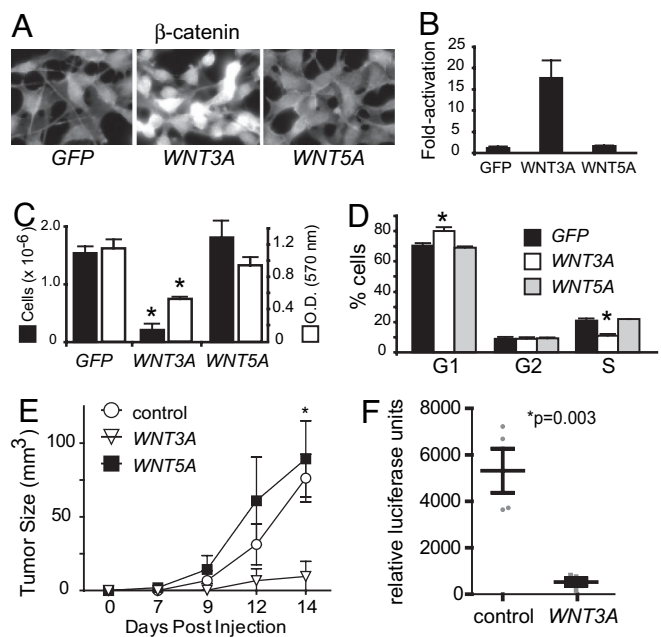


Fig. 3. Wnt/β-catenin activation is associated with decreased proliferation in melanoma. (A) Immunofluorescent staining demonstrates increased nuclear β-catenin in B16 cells expressing WNT3A, but not in cells expressing either GFP or WNT5A. (B) CM from B16:WNT3A cells activated a Wnt/β-catenin reporter in UACC1273 human melanoma cells, indicating that these cells secrete active WNT3A. No activation was seen with CM isolated from B16:GFP or B16:WNT5A cells. (C) Proliferation of B16:GFP, B16:WNT3A, or B16:WNT5A cells was measured by hemacytometry after 6 days of culture (black bars, left y-axis) or by MTT assay after 3 days of culture (white bars, right y-axis). Bars represent the average and standard deviation of 3–6 biological replicates. The inhibition of proliferation seen with WNT3A cells was highly significant by ANOVA with both proliferation assays ($*P < 0.001$). (D) In cell cycle analysis, B16:WNT3A cells were decreased in S phase and increased in G1 compared with B16:GFP or B16:WNT5A cells. Bars indicate the average and standard deviation of 3 biological replicates, and the data shown are representative of 5 individual experiments, each with at least 3 biological replicates per condition. The changes observed in B16:WNT3A cells are extremely significant by ANOVA ($*P < 0.001$). (E) Tumor grafts demonstrate that B16 cells expressing WNT3A form smaller tumors than cells expressing GFP or WNT5A. Data are expressed as mean and standard deviation from 4 mice for each cell line tested. The experiment shown is representative of 4 independent experiments with the same result, with each experiment involving at least 4 mice for each cell line tested. The decrease in tumor size with WNT3A was found to be highly significant by ANOVA at 14 days postimplantation ($*P = 0.004$). (F) Metastases to the popliteal sentinel lymph node bed were evaluated by firefly luciferase assay, demonstrating significantly decreased metastases in tumors expressing WNT3A. Bars represent mean and standard deviation, and the data shown are representative of 4 independent experiments.

paralleled in human cell lines (Fig. S3B and C). Cell cycle analysis revealed that B16:WNT3A cells exhibit an increased population in G1, with a decreased population in S phase, compared with either B16:GFP or B16:WNT5A cells (Fig. 3D). Furthermore, in vivo tumor grafts of B16:WNT3A cells exhibit decreased tumor growth (Fig. 3E) compared with either B16:GFP or B16:WNT5A cells, along with decreased metastasis (Fig. 3F). No differences in apoptosis were seen in tumor grafts by TUNEL staining (data not shown). Together, these data suggest that activation of Wnt/β-catenin signaling correlates with decreased proliferation of melanoma cells.

Activation of Wnt/β-catenin signaling by the GSK-3 inhibitors lithium chloride or 6-bromoindirubin-3'-oxime (BIO) also results in decreased proliferation of cultured B16 (Fig. S4) and human melanoma cells (Fig. S3D), supporting the hypothesis that decreased proliferation in these cells is due to activation of

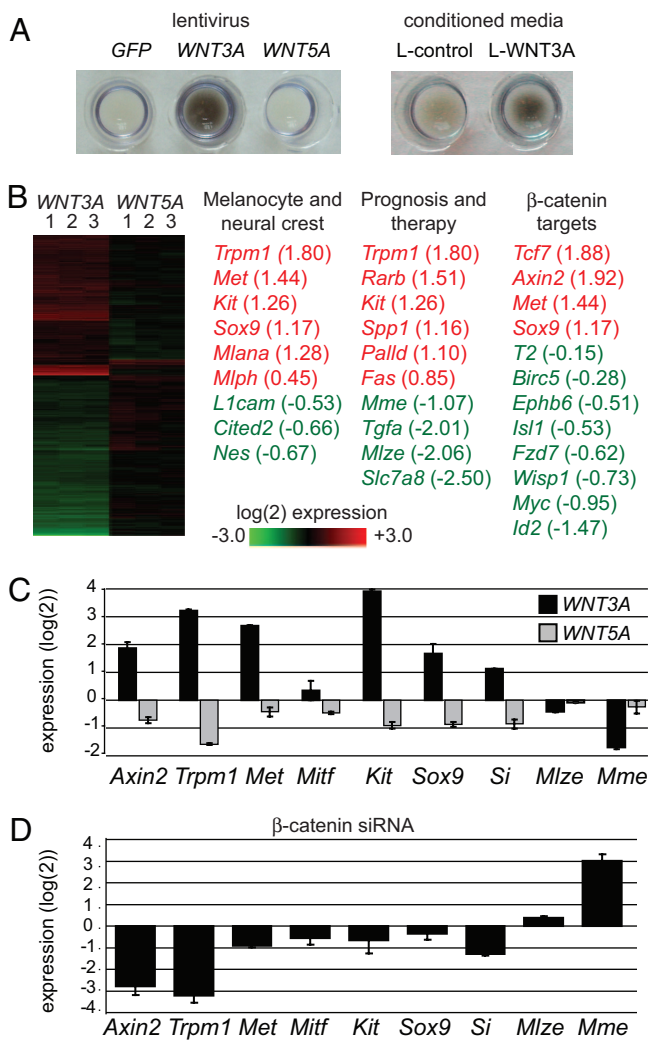


Fig. 4. Activation of Wnt/ β -catenin signaling alters cell fate in melanoma cells. (A) B16:GFP, B16:WNT3A, or B16:WNT5A cells were isolated at equivalent confluency, spun down, and photographed in a 96-well plate, demonstrating the marked difference in pigmentation seen in melanoma cells expressing WNT3A. Increased pigmentation also was seen in B16 cells treated for 4 days with WNT3A-conditioned L-cell media (L-WNT3A) compared with control L-cell media (L-control). (B) Whole genome expression profiles of B16:WNT3A or B16:WNT5A cells were initially compared with gene expression in B16:GFP cells, with 3 biological replicates analyzed for each cell line. The heatmap illustrates the differences between the most significant regulated genes in B16:WNT3A cells compared with B16:WNT5A cells by subsequent unpaired *t*-test. Genes that were among the most significantly regulated in B16:WNT3A cells are listed with their normalized fold-change [log (2)] compared with B16:GFP cells shown in parentheses; these include known Wnt/ β -catenin targets, genes involved in melanocyte and neural crest differentiation, and genes implicated in melanoma prognosis and therapy. (C) Various genes were selected for validation using quantitative RT-PCR (qRT-PCR), including genes implicated in melanocyte differentiation (*Met*, *Kit*, *Sox9*, *Mitf*, *Sl/Gp100*) and melanoma biology (*Trpm1*, *Kit*, *Mme*, *Mlze*), as well as known Wnt target genes (*Axin2*, *Met*, *Sox9*). All genes that were up-regulated in B16:WNT3A cells by transcriptional profiling were up-regulated by qRT-PCR, whereas genes that were down-regulated in B16:WNT3A cells on the array (*Mlze*, *Mme*) also were down-regulated by qRT-PCR. Genes up-regulated in B16:WNT3A cells were universally down-regulated in the B16:WNT5A cells, providing evidence that WNT5A can antagonize transcription of Wnt/ β -catenin gene targets in melanoma cells, even in the absence of WNT3A. Data are expressed as log (2)-transformed fold-change (with standard error) compared with B16:GFP cells and are representative of 3 or more experiments with similar results. (D) Gene changes induced in B16:WNT3A cells were antagonized on treatment with β -catenin siRNA (20 nM) compared with control siRNA (20 nM). Data are expressed as log (2)-transformed fold-change (with standard error) in cells treated with β -catenin siRNA compared with control siRNA.

Wnt/ β -catenin signaling. Our observation that this decreased proliferation cannot be appreciably rescued by either the soluble antagonist Dickkopf-1 (DKK1) or β -catenin-targeted siRNA (Fig. S5) suggests that this cellular change may reflect a commitment to an altered cell fate, which would be consistent with Wnt/ β -catenin signaling regulating cell fate in other contexts as well (36).

Activation of Wnt/ β -Catenin Signaling Up-Regulates Markers of Melanocyte Differentiation. We found that activation of Wnt/ β -catenin signaling in B16-F1 melanoma cells by either lentiviral transduction of WNT3A or treatment of cells with CM containing WNT3A results in increased pigmentation not seen in cells transduced with lentivirus encoding GFP or WNT5A (Fig. 4A, Left) or cells treated with control CM (Fig. 4A, Right). These phenotypic changes, coupled with the observed decreased proliferation and altered cell cycle profile (Fig. 3C and D), led us to hypothesize that activation of Wnt/ β -catenin signaling leads to changes in cell fate.

To test a prediction of this hypothesis, we performed genome-wide transcriptional profiling to determine whether expression of WNT3A in lentiviral-transduced B16-F1 cells alters the expression of genes reflecting cell fates. Using gene expression in B16:GFP cells as a reference, we established profiles of genes regulated in B16:WNT3A and B16:WNT5A cells, and then further identified genes that exhibited the highest variance between these 2 groups to focus on genes regulated by WNT3A. Among the most highly significant genes elevated by WNT3A (Fig. 4B) are *Axin2* (35) and *Tcf7* (37), which are direct targets of Wnt/ β -catenin signaling; *Mme* and *Mlze*, down-regulated genes previously linked to melanoma progression (38, 39); *Mitf*, which is linked to pigment cell fate; and *Trpm1*, *Met*, *Sox9*, and *Kit*, which are highly expressed during melanocyte and neural crest development (40). The complete list of significantly regulated genes is presented in Dataset S1. To confirm the array data, we measured levels of selected transcripts by quantitative RT-PCR (Fig. 4C). We found that changes in gene expression are antagonized by β -catenin siRNA, confirming that the effects of WNT3A on gene expression are specific (Fig. 4D). Thus, the transcriptional profiling supports the hypothesis, evident from visual examination of cells (Fig. 4A), that activation of Wnt/ β -catenin signaling by WNT3A promotes the adoption of characteristics of melanocyte differentiation by melanoma cells.

Previous studies reporting increased expression of WNT5A observed in later-stage, more aggressive melanomas have focused on the potential role of noncanonical Wnt signaling in regulating cell motility (21–24, 41, 42). Besides regulating cell motility, WNT5A antagonizes Wnt/ β -catenin signaling in developmental models and other contexts (4). Consistent with this activity, we found that WNT5A expression antagonizes the expression of genes regulated by WNT3A (Fig. 4C). This finding suggests that the antagonism of Wnt/ β -catenin signaling by WNT5A may contribute to the loss of Wnt/ β -catenin homeostasis seen with melanoma progression.

Discussion

The exact role of Wnt/ β -catenin signaling in melanoma remains controversial, although previous reports suggesting that activation of Wnt/ β -catenin signaling can promote proliferation in different cancers, including in cultured melanoma cells (36, 43), has led to speculation that this pathway may be involved in early aspects of melanoma formation and progression through regulation of tumor growth. In support of the idea that Wnt/ β -catenin activation does not necessarily promote proliferation in melanocytic cells, a recent study found that forced transgenic expression of a stabilized constitutively active β -catenin mutant (β -cat^{STA}) in mice did not increase proliferation of melanocytes or precursor melanoblasts (44). That study also found that restrict-

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