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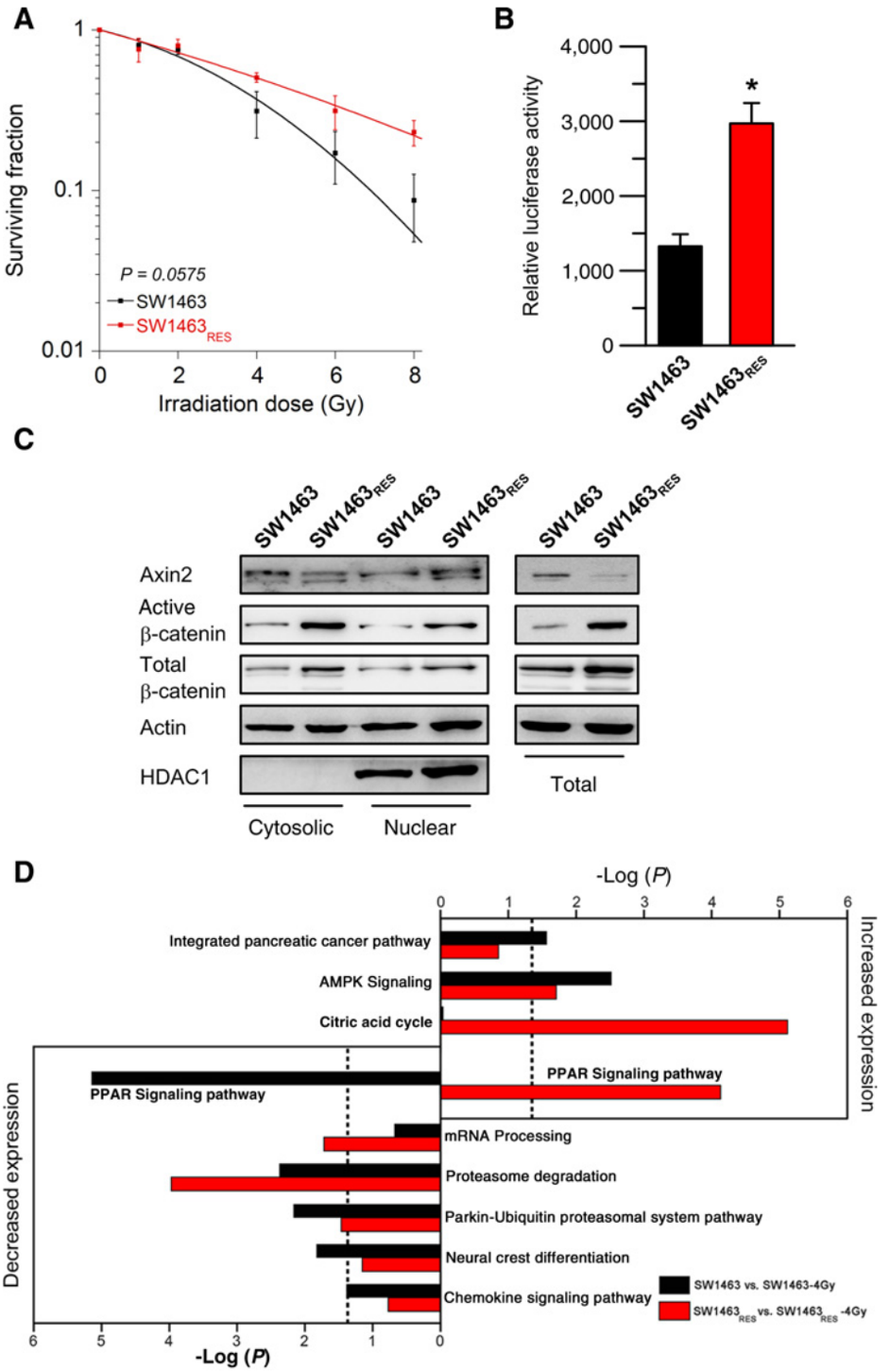

Figure 4.

Isogenic radiation-resistant colorectal cancer cells show elevated Wnt/β-catenin signaling activity. **A,** SW1463 cells were repetitively irradiated with 2 Gy (total dose of 68 Gy), which resulted in a cell population ($SW1463_{RES}$) with a decreased sensitivity to irradiation ($P = 0.0575$; ANOVA model). **B,** This increased treatment resistance was accompanied by a significantly increased TCF/LEF transcriptional activity ($P = 0.002$). **C,** Protein levels of both active and total β-catenin were elevated in radiation-resistant $SW1463_{RES}$ cells. Proteins were isolated as cytosolic and nuclear fractions (left) and whole protein lysates (right). Each experiment was repeated three times. Data are displayed as mean values, $n = 3$, error bars ± SEM. **D,** $SW1463_{RES}$ cells activate distinct pathways in response to a single dose of 4 Gy of X-ray compared with SW1463 cells. Pathway analysis is based on gene expression profiles of three biological replicates of each condition (SW1463, SW1463-4 Gy, $SW1463_{RES}$, $SW1463_{RES}$-4 Gy).

Radioresistant colorectal cancer cells show deregulated RNA expression profiles and activate different pathways in response to irradiation

To elucidate how Wnt/β-catenin signaling mediates resistance, we established gene expression profiles of SW1463 and radioresistant SW1463 ($SW1463_{RES}$), both prior to and 6 hours after exposure to a single dose of 4 Gy (Supplementary Fig. S5B). As expected, we found a significant ($P = 0.05$) overexpression of Wnt/β-catenin signature genes in $SW1463_{RES}$ compared with SW1463, including increased expression of LEF1, part of the TCF/LEF1 transcription factor complex, and WNT5B (Supplementary Table S6A).

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To now assess transcriptional changes in response to radiation and to detect different activation of signaling pathways, we first identified genes differentially expressed between SW1463 cells and SW1463 cells 6 hours after exposure to 4 Gy (SW1463-4 Gy; Supplementary Table S6B). Next, we profiled our radiation-resistant SW1463 cells, prior to ($SW1463_{RES}$) and 6 hours after exposure to 4 Gy ($SW1463_{RES}$-4 Gy; Supplementary Table S6C). Both populations responded to radiation with a significant up- and downregulation of numerous genes and pathways, which can be found in Fig. 4D; Supplementary Tables S6B and S6C. In contrast to SW1463 cells, which responded to radiation with 4 Gy with significantly decreased expression of genes belonging to the PPAR signaling, we observed increased expression of PPAR pathway genes in $SW1463_{RES}$ in response to radiation with 4 Gy (Fig. 4D). In addition, $SW1463_{RES}$ cells responded with overexpression of genes belonging to metabolic pathways, such as AMPK signaling or the citric acid cycle (Fig. 4D).

Discussion

Preoperative chemoradiotherapy followed by radical surgical resection represents the standard of care for patients with locally advanced rectal cancer (4, 11, 26). However, the response of individual tumors to preoperative multimodal treatment is highly heterogeneous and ranges from complete clinical response to absence of any histopathologic tumor regression (complete resistance). This poses a clinical dilemma, because patients with resistant tumors are exposed to the potential side effects of chemotherapy and irradiation with no clear benefit. It is therefore critical to uncover mechanisms and pathways of treatment resistance for the identification of strategies to increase the fraction of patients with rectal cancer who benefit from multimodal neoadjuvant treatment (10).

In an attempt to identify novel molecular targets and pathways that may be manipulated to sensitize tumors to chemoradiotherapy, we previously demonstrated that the Wnt transcription factor TCF7L2 is overexpressed in chemoradiotherapy-resistant rectal cancers (8). Subsequently, we showed that RNAi-mediated silencing of *TCF7L2* sensitizes colon and rectal cancer cell lines to chemoradiotherapy (9). This radiosensitization was the consequence of a transcriptional deregulation of Wnt/TCF7L2 target genes, and a compromised DNA double strand break repair. In addition, silencing of TCF7L2 resulted in an increased fraction of cells in the G_2–M phase of the cell cycle, which is known for increased vulnerability to radiation-induced DNA damage (27). However, it remained unclear whether this effect was a TCF7L2-inherent function or Wnt/β-catenin signaling-dependent. In this study, we confirm that the Wnt/β-catenin pathway mediates resistance of colorectal cancer cells to irradiation and 5-FU–based chemoradiotherapy.

We demonstrated that inhibition of β-catenin, either mediated through siRNAs or the small-molecule inhibitor XAV-939, sensitizes colorectal cancer cells to irradiation. This adds weight to the growing body of evidence suggesting that Wnt/β-catenin signaling mediates treatment responsiveness, in addition to its central role in tumor development and progression (1, 2). Recently, Cojoc and colleagues established gene expression profiles of prostate cancer cell lines and derived radioresistant clones and discovered that β-catenin regulates aldehyde dehydrogenase (ALDH1A3; ref. 28). RNAi-mediated silencing of β-catenin and *ALDH1A3* led to a pronounced radiosensitization of *a priori* resistant cells (28). Fitting, in our experiment, radiosensitive SW1463 cells responded with a significant downregulation of *ALDH1A3* to radiation. Dong and colleagues demonstrated a role for Wnt/β-catenin signaling in radiation-induced invasion of glioblastoma cells (29). In their model, radiation mediated nuclear accumulation of β-catenin and an upregulation of Wnt/β-catenin downstream genes. Pathway inhibition abrogated the proinvasion effects of radiotherapy (29). Similar to our approach, Ahn and colleagues repeatedly irradiated lung cancer cells to obtain resistant cell populations. Using gene expression profiling, they identified multiple genes that were differentially expressed between resistant cells and their parental cell lines. Wnt/β-catenin pathway genes were the most frequently altered (30). In our model, the increased resistance of $SW1463_{RES}$ was accompanied by elevated levels of active and total β-catenin, and increased TCF/LEF transcriptional activity. Interestingly, several of the genes described by Ahn and colleagues (24) were differentially deregulated in a similar fashion in our model, including many Wnt pathway genes. This points to an involvement of Wnt/β-catenin signaling in radiation resistance in multiple tumor entities. Of note, we previously characterized the chemoradiotherapy sensitivity of 12 colorectal cancer cell lines, which we correlated with gene expression profiles. Importantly, and nicely fitting with the observations reported here, we detected an overrepresentation of Wnt-pathway and Wnt-target genes within this signature of chemoradiosensitivity (15).

From a clinical point of view, one goal is to improve sensitivity to chemoradiotherapy. Of equal importance is to decrease potential side effects of irradiation, specifically, to decrease normal tissue toxicity (31). In this respect, Hai and colleagues demonstrated that transient activation of Wnt/β-catenin signaling prevents radiation-induced damage to salivary glands (32). Zhao and colleagues observed that an upregulation of the Wnt/β-catenin pathway accelerates mucosal repair following radiotherapy-induced oral mucositis (33). Very recently, Chandra and colleagues demonstrated that an activated Wnt/β-catenin pathway blocks radiation-induced apoptosis in osteoblasts through enhanced DNA repair, suggesting that Wnt agonists may be clinically used to block radiation-induced osteoporosis (34). In a similar attempt, we stimulated phenotypically "normal" RPE-1 cells, which are highly sensitive to irradiation, with Wnt-3a, a physiologic ligand of the Frizzled receptor family. Treatment with Wnt-3a resulted in a strong activation of Wnt/β-catenin signaling, with increased expression levels of both Axin2 and β-catenin, accompanied by a approximately 800-fold increase of TCF/LEF reporter activity. Importantly, however, Wnt-3a stimulation resulted in significantly increased resistance to irradiation, while inhibition of β-catenin by XAV-939 sensitized RPE-1 to irradiation. A similar effect was observed through overexpression of constitutively active (S33Y-mutated) β-catenin in RPE-1 cells, which also resulted in a strong activation of Wnt/β-catenin signaling and which increased radiation resistance. This effect could be rescued by siRNA-mediated silencing of β-catenin. These results further support the notion that Wnt/β-catenin signaling controls responsiveness to chemoradiotherapy, and that this effect is not due to a β-catenin-independent branch of Wnt signaling (23).

However, open questions remain: Firstly, the underlying molecular mechanisms through which the Wnt/β-catenin

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pathway functionally mediates responsiveness to chemoradiotherapy are still not fully understood. Preliminary evidence indicates that Wnt/β-catenin signaling may trigger epithelial to mesenchymal transition (EMT), which has been implicated in increased resistance to radiotherapy (28, 35–38). In our gene expression data, we observed an overexpression of genes associated with EMT in resistant cell populations, underscoring the importance of the Wnt/β-catenin-EMT-axis (data not shown). Recently, Jun and colleagues suggested a role for nonhomologous end joining (NHEJ) in Wnt/β-catenin–mediated radiation resistance (39). By screening DNA repair genes and β-catenin targets, they identified LIG4, whose expression was directly regulated by β-catenin and observed that deregulation of LIG4 mediates resistance of colon cancer cell lines to radiation. However, we did not observe changes in LIG4 expression when comparing SW1463 and Wnt-active radiation-resistant $SW1463_{RES}$. In addition, LIG4 expression was not associated with treatment response in a set of 161 primary rectal cancers treated with 5-FU-based chemoradiotherapy (Emons and colleagues, unpublished data). This leads to the conclusion that, while the activation of LIG4 may represent one possible mechanism through which Wnt/β-catenin signaling controls responsiveness in colon cancer cells, there likely exist other resistance mechanisms in rectal cancer.

Of note, our data suggest a role for PPAR signaling in mediating radiation resistance. We observed increased expression of genes from the PPAR pathway in response to 4 Gy in $SW1463_{RES}$, while SW1463 cells reacted in a completely opposite manner, that is, with a decrease in expression of these genes. It has been previously shown that PPAR is a downstream pathway of Wnt/β-catenin/TCF7L2 signaling (40), and that PPAR signaling, besides its prominent role in providing metabolic advantages for cancer cells (41), prevents radiation-induced cellular damage. For example, a PPAR-gamma agonist has been recently shown to protect normal tissue from radiation injury (42). Finally, the pathway is associated with a poor prognosis in a variety of human carcinomas (43, 44). Therefore, we speculate that PPAR signaling might be a novel mechanism through which Wnt signaling mediates radioresistance.

Our study did not address the question whether Wnt/β-catenin pathway inhibition *in vivo* sensitizes to chemoradiotherapy. However, several studies reported an association between treatment resistance and the expression of members of the Wnt/β-catenin pathway (45, 46). As described above, we previously used pretherapeutic gene expression profiling of tumor biopsies to demonstrate that *TCF7L2* was expressed at higher levels in resistant compared with responsive primary rectal cancers (8). Using IHC analyses, Kriegl and colleagues reported that TCF7L2 expression was a negative prognostic factor associated with a shorter overall survival of colorectal cancer patients (47). Similarly, Gomez-Millan and colleagues demonstrated that overexpression of β-catenin following chemoradiotherapy was associated with a decreased disease-free survival and a poor prognosis of rectal cancer patients (45).

In summary, our data demonstrate that Wnt/β-catenin signaling mediates responsiveness of rectal cancers to chemoradiotherapy. We suggest that targeting Wnt/β-catenin signaling or one of the downstream pathways represent a promising strategy to increase therapeutic responsiveness of rectal cancers to chemoradiotherapy, the standard treatment for patients with locally advanced stages of this disease (4, 11, 26).

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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Analysis and interpretation of data (e.g., statistical analysis, biostatistics, computational analysis): G. Emons, M. Spitzner, N. Auslander, F. Kramer, Y. Hu, T. Beissbarth, H.A. Wolff, M. Rave-Fränk, J. Gaedcke, M. Grade

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