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Lack of anti-tumor activity with the β-catenin expression inhibitor EZN-3892 in the C57BL/6J Min/+ model of intestinal carcinogenesis

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ABSTRACT

Background: Previously, we showed that short-term inhibition of β-catenin expression and reversal of aberrant β-catenin subcellular localization by the selective COX-2 inhibitor celecoxib is associated with adenoma regression in the C57BL/6J Min/+ mouse. Conversly, long-term administration resulted in tumor resistance, leading us to investigate alternative methods for selective β-catenin chemoprevention. In this study, we hypothesized that disruption of β-catenin expression by EZN-3892, a selective locked nucleic acid (LNA)-based β-catenin inhibitor, would counteract the tumorigenic effect of Apc loss in Min/+ adenomas while preserving normal intestinal function.

Materials and methods: C57BL/6J $Apc^{+/+}$ wild-type (WT) and Min/+ mice were treated with the maximum tolerated dose (MTD) of EZN-3892 (30 mg/kg). Drug effect on tumor numbers, β-catenin protein expression, and nuclear β-catenin localization were determined.

Results: Although the tumor phenotype and β-catenin nuclear localization in Min/+ mice did not change following drug administration, we observed a decrease in β-catenin expression levels in the mature intestinal tissue of treated Min/+ and WT mice, providing proof of principle regarding successful delivery of the LNA-based antisense vehicle. Higher doses of EZN-3892 resulted in fatal outcomes in Min/+ mice, likely due to β-catenin ablation in the intestinal tissue and loss of function.

Conclusions: Our data support the critical role of Wnt/β-catenin signaling in maintaining intestinal homeostasis and highlight the challenges of effective drug delivery to target disease without permanent toxicity to normal cellular function.

1. Introduction

Colorectal adenomas are well-established precursor lesions of colorectal cancer (CRC) [1,2]. The overall goal of CRC chemoprevention is to identify treatments that prevent initial adenoma formation, as well as induce adenoma regression, thereby preventing the malignant transformation of precursor lesions. In humans, more than 80% of CRC demonstrate loss of APC function, resulting in stabilization of β-catenin protein and constitutive activation of Wnt target genes [3]. Previously, we showed that short-term inhibition of β-catenin expression and reversal of aberrant β-catenin subcellular localization by the selective COX-2 inhibitor celecoxib resulted in adenoma regression in Min/+ mice, a CRC model [4]. However, long-term administration resulted in tumor resistance, demonstrating that chemopreventive agents may require intermittent dosing to avoid acquired drug resistance or potential toxicity.

Although aberrant Wnt/β-catenin signaling plays a crucial role in CRC progression, there are currently no effective therapeutic agents available that specifically target this pathway [5]. Ongoing research is focused upon small molecular inhibitors that target β-catenin/TCF interactions or promote β-catenin degradation, but concerns regarding Wnt inhibitor toxicity remain a challenge. Prior reports suggested that ablation of β-catenin expression in enterocytes is lethal in mice [6]. However, it remains possible that adenoma regression can be achieved using a sub-lethal dose of an effective β-catenin-suppressing agent that maintains enterocyte function.

Recently, the development of RNAi, which are RNA particles used to inhibit gene expression, offer novel technologies to target protein synthesis for cancer therapy. RNAi can be delivered via two different approaches: small-interfering RNA (siRNA) or microRNA (miRNA). siRNA therapy is a promising direction for drug development given its high specificity and ease of design and syn-

Abbreviations: CRC, colorectal cancer; APC, adenomatous polyposis coli; FAP, familial adenomatous polyposis; MTD, maximum tolerated dose; siRNA, short-interfering RNA; miRNA, microRNA.

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thesis. However, drug delivery and stability *in vivo* continue to offer sizeable challenges before these agents can be considered viable options for patient use [7]. EZN-3982 is a selective locked nucleic acid (LNA)-based agent designed to specifically inhibit β-catenin expression. EZN-3892 utilizes LNA technology to enhance RNA binding affinity, drug potency, and stability *in vivo*.

In this study, we hypothesized that disruption of β-catenin expression by EZN-3892 would counteract the tumorigenic effects of Apc loss in the Min/+ mouse. We determined the maximum tolerated dose (MTD) of EZN-3892 in our mouse model, and examined the effects of this dose on tumor numbers and intracellular signaling networks relevant to tumorigenic β-catenin activity. The goal of these experiments was to yield important information about Apc-associated tumorigenesis and identify a potentially novel chemoprevention agent for colorectal cancer.

2. Materials and methods

2.1. Materials

C57BL/6J-Min/ (Min/+) and $Apc^{+/+}$ (WT) mice were purchased from The Jackson Laboratory at 6-weeks of age and immediately placed on 5% fat, soy-free AIN-76A diet (Research Diets, New Brunswick, NJ). EZN-3892, a LNA-based antisense vehicle targeted to β-catenin, and EZN-3046, a LNA-based scrambled control, were acquired from ENZON Pharmaceuticals, Inc. (Piscataway Township, NJ). Anti-β-catenin antibody was obtained from BD Transduction (San Diego, CA). Anti-lysozyme antibody was obtained from Dako (Carpinteria, CA). Anti-bromodeoxyuridine (BrdU) antibody was from Roche Applied Science. Other reagents were as previously described [8].

2.2. Drug administration and tissue harvesting

Adult mice (2–3 months of age) were administered study drug, scrambled control, or saline via tail vein injection and sacrificed 4 days after the last treatment dose. Mice were weighed, and the small intestine and colon were removed from each animal. The intestinal tracts were washed with PBS, opened longitudinally, and visible tumors were counted by an individual blinded to treatment status. From the proximal portion of the small intestine, tumors were excised, pooled from each mouse, and frozen in liquid N_2. The mucosal surface of the remaining tissue was scraped with a microscope slide and placed in cold PBS. Cell suspensions were washed twice at 4 °C, and cell pellets were frozen in liquid N_2. The distal portion of the small intestine was Swiss-rolled using a cotton-tipped applicator and submerged in formalin for immunohistochemical analysis.

2.3. Immunohistochemistry

Serial sections of intestine were fixed in 10% neutral buffered formalin and embedded in paraffin for analysis, as previously described [9].

2.4. BrdU proliferation assay

For short-term 5-bromo-2′-deoxyuridine (BrdU) incorporation experiments, mice were injected i.p. with 2 mg of BrdU (Sigma) 2 h before sacrifice. Anti-BrdU antibody was used to detect proliferating cells.

2.5. Western blot analysis

Total cell lysates were prepared in the presence of calpain inhibitor 1 and used for protein analysis, as previously described [8]. All experiments were performed using pooled tissues from 12 different mice per genotype (WT, Min/+) and treatment group (EZN-3892, scrambled LNA control, and saline). Mice were treated every 3 days for 9 doses total and sacrificed 4 days after the last dose. Immunoblottings were replicated three times. Band intensity was normalized to the internal β-actin control and compared relative to the untreated control as fold difference using Image J software from the NIH.

2.6. Statistical analysis

Polyp numbers, β-catenin band intensity, and cell counts were compared relative to untreated controls using Student's *t*-test ($p < 0.05$ was considered significant).

3. Results

3.1. Determination of maximum tolerated dose (MTD) of EZN-3892

A toxic dose of EZN-3892 was expected to ablate small intestinal crypts and substantially reduce the plasma membrane and nuclear pools of β-catenin within a week [6]. Therefore, a dose response for this effect should be evident, and the maximum tolerated dose (MTD) will be the highest dose that maintains viability, preserves normal intestinal crypt morphology, but lowers β-catenin expression and cellular distribution. To determine the MTD of EZN-3892, adult WT and Min/+ mice (2 months of age), fed AIN-76A diet from weaning, were injected by tail vein in parallel with a range of EZN-3892 doses (100, 60, and 30 mg/kg; $n = 2$ for each treatment dose and genotype). Drug was administered every third day for a total of 9 doses, as tolerated. Min/+ mice treated with 100 mg/kg of EZN-3892 developed severe side effects following the 4th treatment dose manifested by weight loss, moribund appearance, and death. One Min/+ mouse treated with 60 mg/kg was euthanized following the 3rd treatment due to toxic side effects. The Min/+ mice treated with 30 mg/kg of EZN-3892 tolerated this dose without any apparent adverse effects and were sacrificed after the 9th dose for tissue analysis. In contrast to Min/+, all WT mice tolerated the full range of treatment doses for the duration of the experiment. Treatment was repeated on a similar group of Min/+ mice using a narrower dose range (45, 30 and 15 mg/kg). Immunohistochemical analysis of intestinal sections from these animals showed that the middle dose was again tolerated and was the lowest concentration that visibly reduced β-catenin expression (see below). Thus, a dose of 30 mg/kg is the MTD of EZN-3892 in Min/+ mice.

3.2. Intestinal crypt morphology was preserved following treatment with the EZN-3892 MTD

To investigate the effect of EZN-3892 on small intestinal crypt morphology and β-catenin expression, we performed immunohistochemistry for β-catenin on small intestine from both WT and Min/+ mice treated with the MTD of 30 mg/kg (Fig. 1A). In both genotypes, β-catenin localization at the cell membrane of the small intestinal villi was preserved following treatment with EZN-3892 relative to scrambled control. In treated Min/+ tissue, β-catenin expression at the cell membrane appeared lower relative to untreated tissue (Fig. 1A). Paneth cell differentiation and localization at the crypt base is driven by canonical Wnt signaling, and β-catenin-Tcf4 activates Lysozyme expression [10]. Therefore as a surrogate for Wnt signaling, we also investigated the effect of EZN-3892 compared to the scrambled control on the relative abundance and localization of Paneth cells at intestinal crypt bases (Fig. 1B). Lysozyme staining of sectioned ileum showed a similar

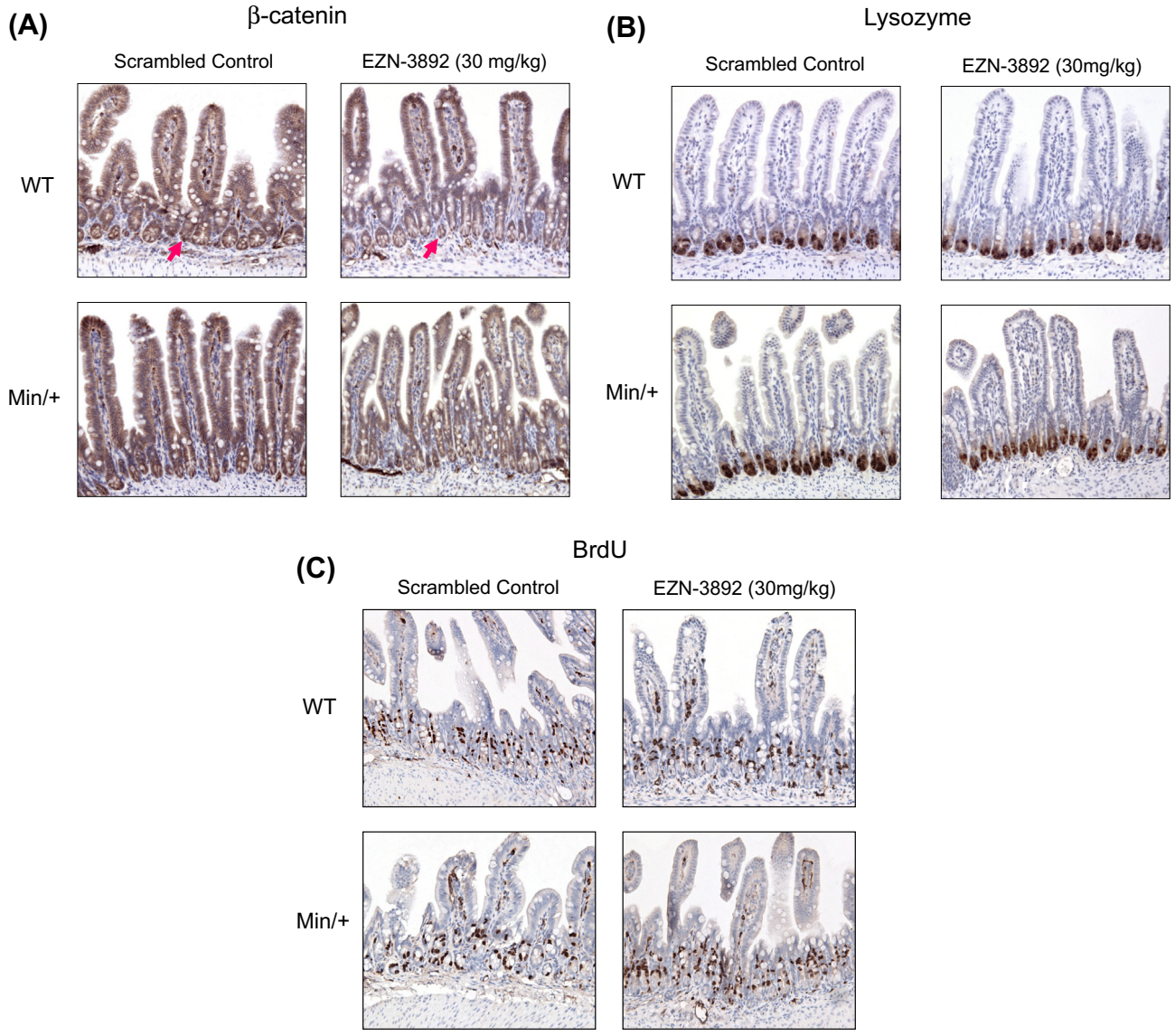

Fig. 1. Effect of EZN-3892 on intestinal crypt morphology. WT and Min/+ mice were treated with 30 mg/kg of EZN-3892 or scrambled control every 3 days and sacrificed after the 9th dose. Immunohistochemical staining for β-catenin demonstrated preservation of membrane localization in both WT and Min/+ mice following either treatment. Nuclear β-catenin staining of Paneth cells was also present (red arrows) (A). Immunohistochemical staining of lysozyme, a Paneth cell marker, showed that Paneth cell localization at the crypt bases was maintained following treatment with EZN or scrambled control (B). Proliferation was conserved in Min/+ and WT mice as demonstrated by BrdU incorporation (C). Representative images were taken using an Olympus BX40 microscope at 10× magnification. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

number and proper positioning of these specialized cells indicating that the Paneth cell maturation program in stem/progenitor cells was not significantly affected by either EZN-3892 or control treatments. Finally, in both EZN and scrambled control groups, proliferation was maintained in Min/+ and WT mice as demonstrated by BrdU incorporation (Fig. 1C).

3.3. EZN-3892 decreased β-catenin expression in mature intestinal tissue of Min/+ mice

To determine the effect of EZN-3892 on β-catenin levels in the small intestine, we performed Western blot analysis on cell lysates prepared from WT, Min/+, and Min/+ tumors treated with 30 mg/kg of EZN-3892, scrambled LNA control, and saline every 3 days for 9 doses total ($n = 12$ mice per genotype and treatment group). Because post-mitotic enterocytes of villi significantly outnumber those of stem/progenitor cells in crypts, blotting data reflect inducible effects on the mature cell population and the tissue overall. Consistent with our immunohistochemistry results, Western blotting revealed that total β-catenin levels were decreased in the mature small intestinal tissue of Min/+ mice treated with EZN-3892 relative to the saline group ($p = 0.007$) (Fig. 2A). Off-target activities resulting from the scrambled control LNA may have occurred since intestinal tissue of Min/+ mice treated with this compound also showed decreased overall β-catenin expression compared to the saline treated group (Fig. 2A). β-Catenin inhibition following treatment with EZN-3892 was less pronounced in WT tissue. Importantly, no differences were observed in β-catenin expression levels among the Min/+ tumor treatment groups (Fig. 2B), consistent with the lack of response in tumor numbers in Min/+ mice treated with EZN-3892 (Fig. 3).

3.4. EZN-3892 did not reduce intestinal tumor counts in Min/+ mice

Short-term inhibition of β-catenin expression and reversal of aberrant β-catenin subcellular localization is associated with

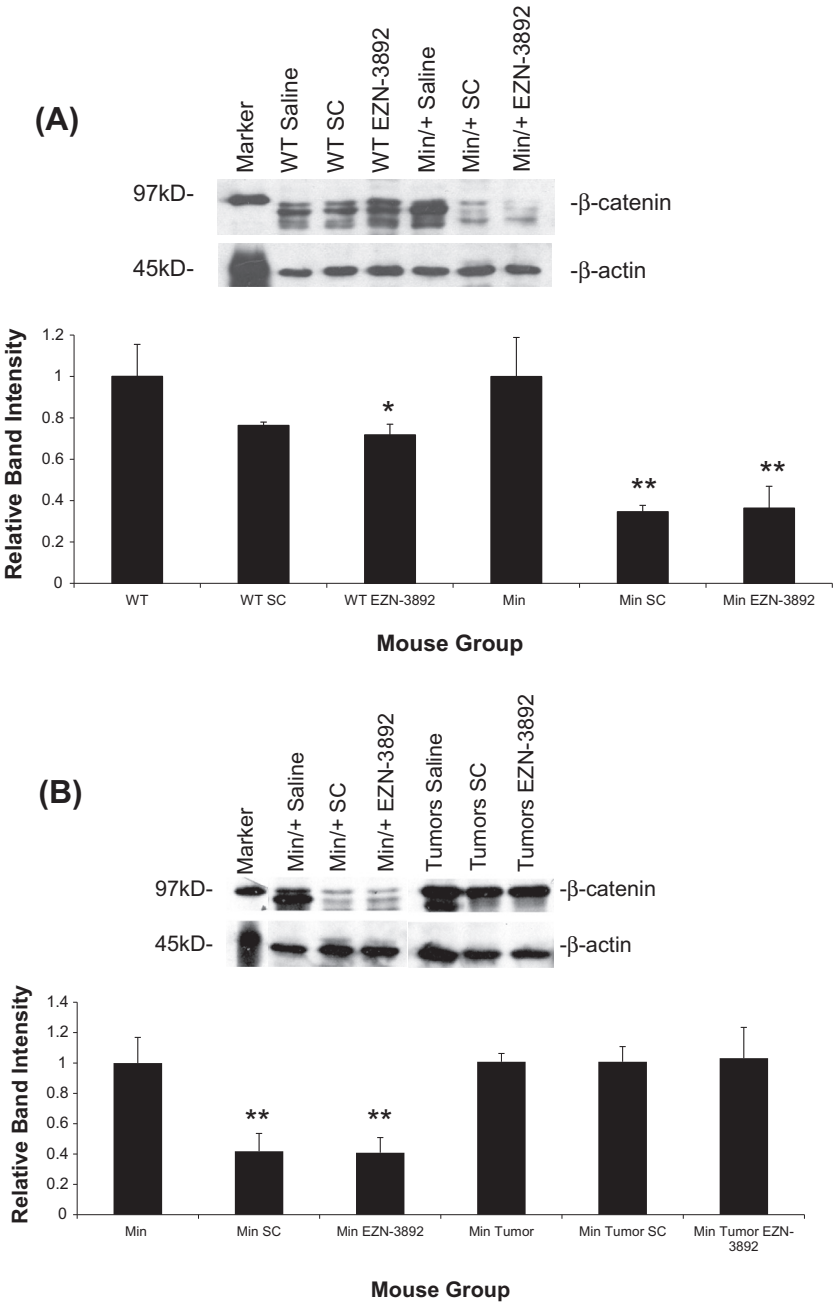

Fig. 2. Effect of EZN-3892 on β-catenin expression in Min/+ mice. For Western blot analysis, lysates were pooled from 12 different mice per genotype (WT/Min/+) and treatment group. Immunoblottings were replicated three times. Band intensity was normalized to the internal β-actin control and compared relative to the untreated control as fold difference using Image J software from the NIH. Treatment with EZN-3892 and scrambled control (SC) decreased overall β-catenin expression in Min/+ enterocytes relative to saline controls (A). In contrast, treatment with EZN-3892 and scrambled control had no effect on overall β-catenin expression in Min/+ tumors (B). * denotes $p < 0.05$ and ** denotes $p < 0.01$ relative to controls.

regression of established tumors and reduced tumor formation [4]. To determine whether disruption of β-catenin expression by EZN-3892 would counteract tumor promotion by Apc loss in Min/+ adenomas, WT and Min/+ mice ($n = 12$ mice per group) were treated with 30 mg/kg of EZN-3892, scrambled LNA control, and saline every 3 days for 9 doses total. Following treatment, mice were sacrificed and small intestinal tumors were counted by both gross and microscopic examination (Fig. 3). Tumors were not observed in WT mice from any treatment group. There was no significant difference in the average number of gross tumors counted in Min/+ mice from the EZN-3892 ($n = 5.3 \pm 4.7$), scrambled control ($n = 6.7 \pm 7.8$), or saline groups ($n = 5.5 \pm 4.7$) (Fig. 3A). Tumor counts performed at 10× magnification on the distal portions of the small intestine also demonstrated no significant differences: EZN-3892 ($n = 11.2 \pm 4.1$), scrambled control ($n = 9.3 \pm 6.3$), or saline groups ($n = 7.0 \pm 4.7$) (Fig. 3B).

3.5. EZN-3892 did not alter nuclear β-catenin expression in Min/+ tumors

Next, we determined whether β-catenin localization was altered in the treated tumors. We performed nuclear β-catenin cell counts (>1000 cells per mouse) in 5 different mice per treatment group. There was no difference in the percentage of positive

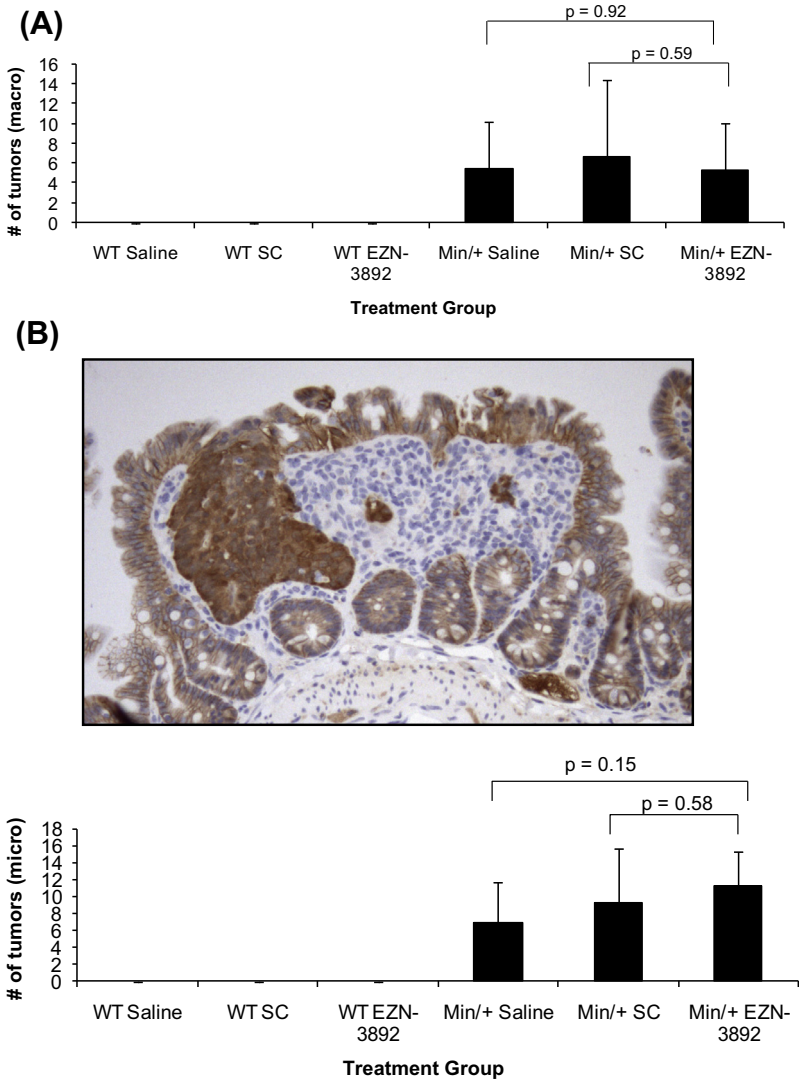

Fig. 3. Effect of EZN-3892 on intestinal polyp numbers in Min/+ mice. WT and Min/+ mice ($n = 12$ mice per group) were treated with 30 mg/kg of EZN-3892, scrambled control (SC), or saline every 3 days and sacrificed after the 9th dose. There were no tumors observed in WT mice. There was no significant difference in macroscopic tumor counts in Min/+ mice treated with EZN-3892 (5.3 ± 4.7), scrambled control (6.7 ± 7.8), or saline (5.5 ± 4.7); $p = 0.59$ for EZN-3892 versus scrambled control and $p = 0.92$ for EZN-3892 versus saline (A). Representative image of small intestinal tumor at 10× magnification stained with β-catenin (B). There was no significant difference in microscopic tumor counts in Min/+ treated with EZN-3892 (11.2 ± 4.1), scrambled control (9.3 ± 6.3), or saline (7.0 ± 4.7); $p = 0.58$ for EZN-3892 versus scrambled control and $p = 0.15$ for EZN-3892 versus saline (B).

nuclear β-catenin cells in the EZN-3892 treated cohort (20.29 ± 5.5%) versus the scrambled control LNA (24.2 ± 6.1%) or saline groups (16.4 ± 6.3%) (Fig. 4). Of note, tumors in Min/+ mice demonstrated markedly elevated cytoplasmic and nuclear β-catenin expression relative to normal adjacent tissue.

4. Discussion

Targeted colorectal cancer (CRC) therapies directed towards specific deregulated signaling pathways have been a mainstay of clinical trials in recent years. Although attempts to standardize the use of surgery, radiotherapy, and systemic chemotherapy for patients is ongoing, controversy still exists regarding selection of the optimal chemotherapeutic regimen and tailored targeted therapy [11]. Agents including bevacizumab and cetuximab, which selectively target vascular endothelial growth factor (VEGF) and epidermal growth factor receptor (EGFR), respectively, have met with limited success in patients with advanced disease. In many cases, cancers developed adaptive drug resistance, resulting in only a short delay in disease progression [12]. Others treatments have targeted the MAPK, PI3K, and angiogenesis/hypoxia pathways, and clinical trials designed to determine efficacy for these drugs are currently underway [12]. Given the multiple molecular pathways involved in driving CRC progression, it is apparent that effective treatment will require a combination of several therapeutic compounds to enable modulation of therapy for the individual patient.

The Cancer Genome Atlas Network recently reported that the Wnt signaling pathway was deregulated in 93% of all human CRC tumors [13]. Despite the fact that greater than 80% of patients with CRC demonstrate loss of APC function, a key negative regulator of β-catenin protein stability, there are no effective therapies available to target the Wnt/β-catenin signaling activities to achieve tumor inhibition *in vivo*. Previously, we showed that celecoxib-mediated inhibition of β-catenin expression and reversal of aberrant β-catenin subcellular localization correlated with adenoma regression in Min/+ mice [4]. Since long-term treatment produced drug resistance and tumor regrowth, we sought to investigate alternative methods for targeting β-catenin in our mouse model.

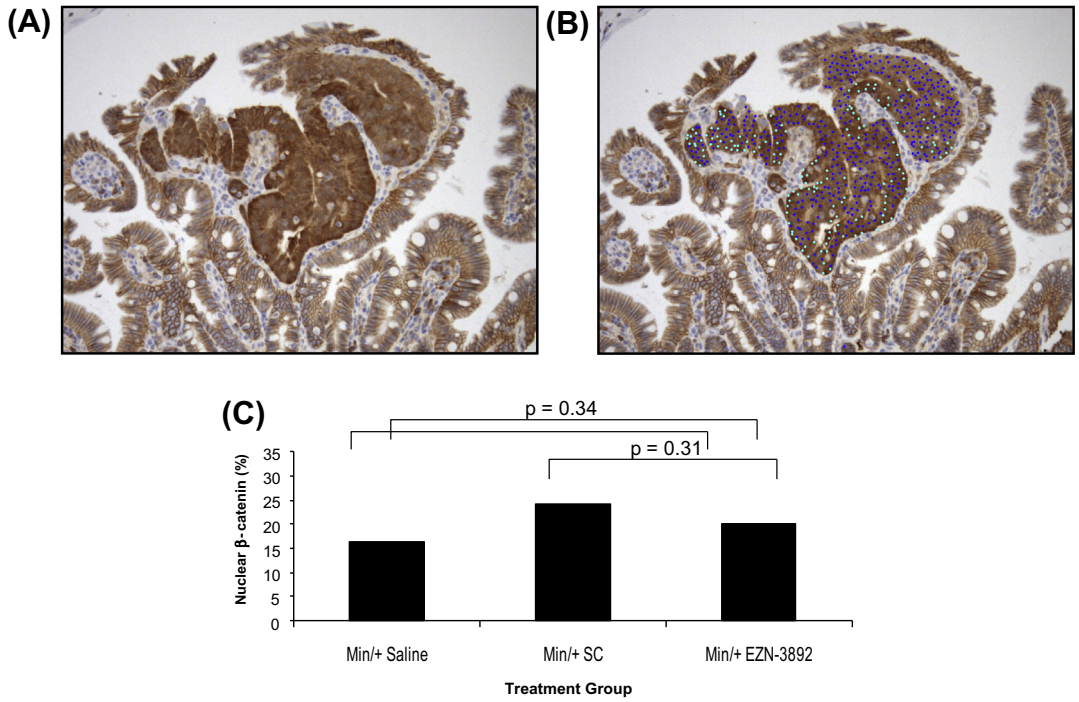

Fig. 4. Effect of EZN-3892 on nuclear β-catenin localization. Min/+ adenomas were stained for β-catenin and analyzed under 10× magnification (A). Image J software from the NIH was used to count nucleated cells (blue dots) and cells containing nuclear β-catenin were marked (green dots). A minimum of 1000 cells were counted per mouse and 5 different mice per treatment group were analyzed (B). There was no significant difference in the percentage of cells with nuclear β-catenin in Min/+ adenomas from mice treated with EZN-3892 (20.2 ± 5.5%) versus scrambled control (24.2 ± 6.1%) or saline (16.4 ± 6.3%); $p = 0.31$ for EZN-3892 versus scrambled control and $p = 0.34$ for EZN-3892 versus saline (C). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

In this study, we examined whether LNA-based antisense knockdown of β-catenin would inhibit tumor formation in Min/+ mice and potentially identify a novel chemoprevention agent for CRC.

Although treatment with EZN-3892 did not result in significant inhibition in tumor numbers, we did observe a decrease in overall β-catenin expression in the mature small intestinal tissue of treated Min/+ mice (Fig. 2A). This decrease in β-catenin expression was also observed by immunohistochemistry in treated Min/+ mice (Fig. 1A). In contrast, at the maximum tolerated dose of 30 mg/kg, β-catenin levels in tumors (Fig. 2B) and nuclear localization of β-catenin in adenomas (Fig. 4) were unchanged. We cannot rule out that a higher dose of EZN-3892 would produce tumor regression but all Min/+ mice treated with 60 mg/kg demonstrated lethal side effects, likely secondary to β-catenin ablation in the normal gut epithelium. This evidence for toxicity is supported by Fevr et al. who reported that inducible β-catenin ablation in enterocytes resulted in terminal differentiation of stem/progenitor cells, loss of intestinal homeostasis, and rapid death in mice [6]. Similarly, Chen et al. showed loss of intestinal stem/progenitor cell function and gross alterations in tissue architecture in zebrafish treated with small molecule inhibitors of the Wnt pathway [14]. Of note, inhibition of stem cell function appeared to be transitory once treatment was stopped, suggesting that intermittent drug treatments may effectively inhibit pathologic Wnt/β-catenin activity without permanently blocking the capacity for self-renewal.

Although β-catenin expression in the mature intestinal tissue of WT mice decreased following treatment, the effect was partial and less robust than observed in Min/+ mice (Fig. 2A). Consistent with this finding, WT mice tolerated higher doses of EZN-3892 (up to 100 mg/kg) that were toxic in Min/+ mice. Our data suggest that the intestinal epithelium of Min/+ mice is more sensitive to alterations in mucosal homeostasis relative to WT or intestinal tumors, which were resistant to drug effect. We previously reported that histologically normal Min/+ intestinal epithelium exhibited differences in proliferation, apoptosis, and crypt-villus migration relative to WT tissue [15,16]. It is conceivable that intrinsic changes in Min/+ mice render the intestine less capable of repair following damage to stem/progenitor cells induced by drugs targeting the Wnt pathway. enterocytes after drug treatment. This idea has implications for patients since CRC or colorectal adenomas likely exist in a deranged non-tumor intestinal microenvironment which may also respond to drugs with differential toxicity relative to disease-free tissue counterparts.

Despite our negative findings, other studies investigating small molecular inhibitors of the Wnt/β-catenin pathway in CRC showed promising initial results. Lee et al. found that a small molecular inhibitor of Wnt/β-catenin inhibited cellular proliferation and transcription of downstream target genes in colon cancer cell lines [17]. Similarly, another study demonstrated that β-catenin inhibition using shRNA significantly decreased proliferation in HCT116 and Ls174t colon cancer cell lines [18]. In an animal model, Gwak et al. reported that the small molecule CGK062 promoted PKCα-mediated β-catenin degradation resulting in tumor suppression in xenograft mice [19]. Our findings offer a similar proof of principle demonstration of vehicle uptake with subsequent reduction in β-catenin expression in target tissue, albeit with toxic effects at higher doses. However, our studies were conducted using a mouse model of human CRC with adenomas of these mice characteristically exhibiting loss of the Apc^{+} allele (rendering them Apc-null) as well as marked over-expression and aberrant nuclear localization of β-catenin [20]. Hence, the Min/+ mouse is a more appropriate model for studying both efficacy and toxicity of small molecule inhibition of the Wnt/β-catenin pathway in a whole tissue context compared to *in vitro* or xenograft studies. Further investigation of downstream Wnt targets that cause tumor regression without affecting stem cell viability is warranted.

In conclusion, our data demonstrate that inhibition of the Wnt/β-catenin pathway in Min/+ enterocytes resulted in decreased

intestinal β-catenin expression levels but no significant reduction in tumor numbers following treatment with the MTD of the LNA-based antisense EZN-3892. In contrast, Min/+ tumors demonstrated resistance to drug effects at the MTD. Our findings suggest that Wnt/β-catenin signaling plays an important role in maintaining normal intestinal homeostasis and that significant β-catenin expression loss results in lethal complications in Min/+ mice. Effective use of targeted chemoprevention will need to balance selective drug delivery with minimal toxicity to normal cellular functions.

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Disclosures

The authors have no financial conflicts to disclose.

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