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WNT signaling controls expression of pro-apoptotic *BOK* and *BAX* in intestinal cancer

Jurrit Zeilstra [a], Sander P.J. Joosten [a], Felix M. Wensveen [b], Mark C. Dessing [a], Denise M. Schütze [a], Eric Eldering [b], Marcel Spaargaren [a], Steven T. Pals [a,*]

[a] Department of Pathology, Academic Medical Center, University of Amsterdam, The Netherlands
[b] Department of Experimental Immunology, Academic Medical Center, Amsterdam, The Netherlands

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ABSTRACT

In a majority of cases, colorectal cancer is initiated by aberrant activation of the WNT signaling pathway. Mutation of the genes encoding the WNT signaling components adenomatous polyposis coli or β-catenin causes constitutively active β-catenin/TCF-mediated transcription, driving the transformation of intestinal crypts to cancer precursor lesions, called dysplastic aberrant crypt foci. Deregulated apoptosis is a hallmark of adenomatous colon tissue. However, the contribution of WNT signaling to this process is not fully understood. We addressed this role by analyzing the rate of epithelial apoptosis in aberrant crypts and adenomas of the $Apc^{Min/+}$ mouse model. In comparison with normal crypts and adenomas, aberrant crypts displayed a dramatically increased rate of apoptotic cell death. Expression profiling of apoptosis-related genes along the crypt-villus axis and in *Apc* mutant adenomas revealed increased expression of two pro-apoptotic Bcl-2 family members in intestinal adenomas, *Bok* and *Bax*. Analysis of the colon of familial adenomatous polyposis (FAP) patients along the crypt-to-surface axis, and of dysplastic crypts, corroborated this expression pattern. Disruption of β-catenin/TCF-4-mediated signaling in the colorectal cancer cell line Ls174T significantly decreased *BOK* and *BAX* expression, confirming WNT-dependent regulation in intestinal epithelial cells. Our results suggest a feedback mechanism by which uncontrolled epithelial cell proliferation in the stem cell compartment can be counterbalanced by an increased propensity to undergo cell death.

1. Introduction

The continuous self-renewal of the intestinal epithelium is a tightly controlled dynamic process. Crypt base columnar cells in the intestinal crypts, recently identified as intestinal stem cells, divide daily and their daughter cells constitute a transit-amplifying compartment of rapidly dividing epithelial progenitors. These, in turn, differentiate while migrating upwards and are eventually shed into the lumen [reviewed by van der Flier [1]]. The WNT signaling pathway plays a critical role in the maintenance of the stem cell and transit-amplifying compartments [2]. Active WNT signaling promotes the nuclear translocation of cytoplasmic β-catenin, the stability of which is regulated by a destruction complex containing adenomatous polyposis coli (APC), AXIN and GSK3β. When translocated to the nucleus, β-catenin activates the transcription of target genes through its interactions with TCF/LEF-family transcription factors. The requirement for active WNT signaling in the crypt compartment is illustrated by the absence of stem cells and proliferative crypts in mice lacking either Tcf-4 [3] or β-catenin [4]. In contrast, mutation of the genes encoding APC or β-catenin causes constitutively active β-catenin/TCF-mediated transcription and initiates the formation of aberrant crypts or microadenoma (MA), the earliest identifiable colorectal cancer precursor lesions [5,6].

Besides promoting cell proliferation and tissue expansion, WNT signaling has also been linked to the regulation of apoptotic cell death. For instance, active WNT signaling drives apoptosis of neuronal cells in the retina of *Drosophila* [7] and a similar relation between active WNT signaling and apoptosis has also been reported in the intestinal crypt compartment. For example, epithelial crypt cells expressing a constitutively active LEF1/β-catenin fusion protein were highly sensitive to apoptosis [8]. Moreover, the conditional deletion of APC in all intestinal epithelial cells of adult mice strongly increased the rate of apoptotic cell death in the proliferating epithelial cell fraction [9,10]. In addition, in comparison to cells of the basal crypt compartment, the epithelial cells lining the villi of the small intestine and intercrypt tables of the colon, where WNT signaling is low, were relatively resistant to apoptosis [11]. The Bcl-2 family of pro- and anti-apoptotic proteins regulates the critical balance between survival and controlled cell death in the intestinal mucosa [reviewed by Watson [12]]. Interestingly,

* Corresponding author. Address: Department of Pathology, Academic Medical Center, University of Amsterdam, Meibergdreef 9, 1105 AZ Amsterdam, The Netherlands. Fax: +31 20 5669523.
E-mail address: s.t.pals@amc.uva.nl (S.T. Pals).

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inactivation of the tumor suppressor protein p53, a key regulator of apoptosis in epithelial cells, had no effect on the rate of apoptosis in crypts and adenomas [13,14], suggesting p53-independent pro-apoptotic signaling upon neoplastic transformation of intestinal crypts. These findings indicate a causal relationship between (aberrant) activation of WNT signaling and increased apoptosis sensitivity. Despite these findings, however, the effects of WNT/β-catenin signaling on the apoptotic program have remained largely unclear. In the current study, we explored this relationship by analyzing apoptotic cell death at the earliest stages of WNT-driven intestinal tumorigenesis in tumor prone $Apc^{Min/+}$ mice and FAP patients. Our results demonstrate an important function of WNT signaling in controlling the expression levels of two pro-apoptotic Bcl-2 family members, *BOK* and *BAX*, in intestinal epithelial cells during tumorigenesis and suggest a feedback mechanism by which uncontrolled epithelial cell proliferation in the stem cell compartment can be counterbalanced by an increased propensity to undergo cell death.

2. Materials and methods

2.1. Immunohistochemistry and quantification of apoptotic cells

$Apc^{Min/+}$ mice were obtained from the Jackson Laboratory and were sacrificed at 8 weeks of age. Small intestine was isolated and immunohistochemistry was performed on paraffin-embedded tissue sections as described in Zeilstra et al. [15]. The antibody used was rabbit anti-cleaved caspase-3, Asp175 (Cell Signaling Technology). Multiple images of crypts, microadenomas (<0.25 mm) and adenomas (>1 mm) were acquired and total epithelial cell numbers as well as apoptotic epithelial cells were quantified using ImageJ software [16]. Clusters of apoptotic fragments were scored as one cell death event and Apoptotic Index (AI), defined as the proportion of active caspase-3 positive cells per 100 epithelial cells, was determined as previously described [11]. Student's *t* test was used for statistical analysis. Samples of human colon were obtained according to standard medical ethical procedures of the Academic Medical Center. Paraffin-embedded colonic sections were stained using primary mAb mouse anti-human BAX, Ab-1 (Thermo Fisher Scientific). Antibody binding was visualized using the Powervision poly-HRP detection system (ImmunoVision Technologies) and DAB+ (Dako).

2.2. Laser-aided microdissection

Patients were diagnosed with FAP with 100 or more colorectal adenomas (two males, two females, median age: 30 years, range: 22–44 years). Sections of snap-frozen tissue were counter-stained with hematoxylin and digitally scanned with a Veritas Microdissection System (Molecular Devices Corporation). Epithelial cells were cut and collected into Capsure Macro LCM caps. RNA was isolated using the PicoPure RNA Isolation Kit (Molecular Devices Corporation) according to the manufacturer's protocol. RT-PRC primers used are listed in Supplementary Table 1. Student's *t* test was used for statistical analysis.

2.3. RT-MLPA

MLPA method was described by Schouten et al. [17]. RT-MLPA was performed as described earlier by Eldering et al. [18]. Apoptosis related gene expression was analyzed using the SALSA RT-MLPA kit R011 (MRC-Holland). Amplicons were quantified using an ABI 3100 Avant capillary electrophoresis system (Applied Biosystems) after addition of the GeneScan-500 ROX size standard. Data were analyzed using Genotype and GeneScan software (Applied Biosystems). The sum of all data values was set at 100% to correct for fluctuations in total signal between samples, and individual values were calculated relative to the 100% value. Results were statistically analyzed using Student's *t* test and *P*-values were corrected for multiple comparisons according to the method of Benjamini and Hocherg. Values less than .05 were considered statistically significant.

2.4. Cell culture

Inducible dominant-negative TCF-4 Ls174T cells were a generous gift of Clevers and van de Wetering [19]. Cells were cultured in RPMI (10% FCS) and expression of dnTCF-4 was induced with 1 μg/ml doxycyclin for 24 h or vehicle as control.

2.5. Real-time reverse transcription-PCR

Quantitative reverse transcription-PCR (qRT-PCR) runs were performed on a Roche LightCycler 1.5 using FastStart DNA Master SYBR Green I kit (Roche Molecular Systems). Results were analyzed using LinReg PCR analysis software (version 7.5; [20]). Expression was normalized over *β-actin* expression.

3. Results

3.1. Intestinal microadenomas display increased sensitivity to apoptosis after loss of functional APC

To investigate the rate of apoptosis in microadenomas and adenomas initiated by APC mutations we made use of the $Apc^{Min/+}$ model. These mice carry an *Apc* allele with a truncating mutation at codon 850 and, similar to FAP patients, spontaneously develop multiple intestinal lesions throughout their intestinal tract [21]. Apoptotic intestinal epithelial cells were visualized by immunohistochemical staining of small intestinal tissue sections of 8 weeks old $Apc^{Min/+}$ mice using an antibody which recognizes active caspase-3 (Fig. 1A–C). Examination of the normal crypt compartment revealed the presence of low numbers of apoptotic enterocytes (Apoptotic Index (AI): 0.023 ± 0.002 (mean ± SE, $n = 14$). This small but constant rate of apoptosis is in line with previously reported data [22]. Strikingly, intestinal microadenomas displayed an over 150-fold increase in apoptosis compared to normal crypts (AI of 3.78 ± 0.35, $P < 0.001$; Fig. 1D). The AI of full size intestinal adenomas was 0.72 ± 0.06 (mean ± SE; $n = 50$), a more than 30-fold increase compared to that of normal crypts ($P < 0.001$) but, interestingly, a more than 5-fold reduction when compared to the AI of microadenomas ($P < 0.001$; Fig. 1D). We conclude that intestinal adenoma formation, caused by loss of functional APC, is accompanied by strongly elevated levels of apoptosis. Hence, these findings suggest that enhanced WNT signaling potentiates mitochondrial apoptosis in the intestinal epithelium during intestinal tumorigenesis.

3.2. Increased gene expression of pro-apoptotic Bok and Bax in Apc mutant adenomas

We next set out to identify putative WNT-regulated apoptosis-related genes in the intestinal mucosa. This was addressed by performing gene expression profiling of laser-capture microdissected intestinal epithelial cells isolated from normal duodenal crypts, adjacent villi, and from distant duodenal adenomas of 8 weeks old $Apc^{Min/+}$ mice ($n = 4$). For this purpose, we employed RT-Multiplex Ligation-dependent Probe Amplification (RT-MLPA), a PCR-based method that allows for the relative quantification of over 35 apoptosis-related genes in small amounts of RNA [18]. (For a complete list of genes and results: see Supplementary Table 2).

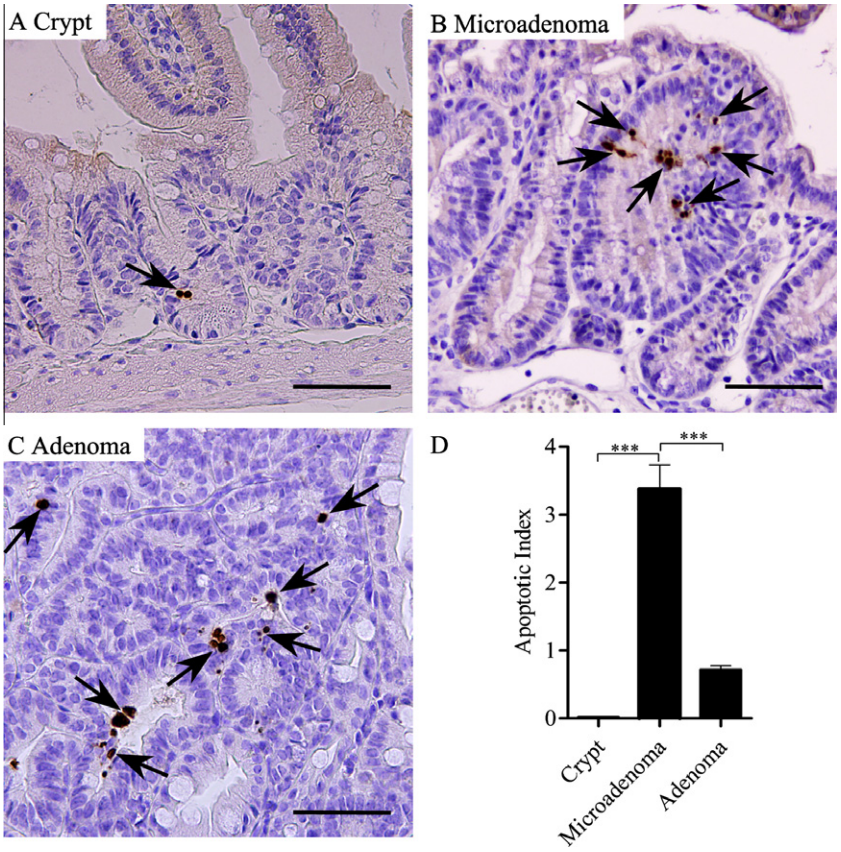

Fig. 1. Increased apoptosis in intestinal microadenomas of $Apc^{Min/+}$ mice. Apoptotic epithelial cells in the intestinal mucosa visualized by immunohistochemical staining for active caspase-3 (arrows). (A) Normal small intestinal crypt. (B) Microadenoma. (C) Adenoma. (Scale bars represent 50 μm). (D) Apoptotic index of crypts ($n = 14$), microadenomas ($n = 32$) and adenomas ($n = 50$) (***$P < 0.001$).

We identified 10 genes with a significant ($P < 0.05$), and at least 2-fold change in expression level in villi (low WNT signaling) compared with crypts (active WNT signaling) (Table 1). The top down-regulated gene along the crypt-villus axis was *Birc5* (also known as *Survivin*) (>14-fold, $P < 0.001$), a member of the inhibitory of apoptosis (IAP) gene family with an essential function in cell division [23]. *Birc5* is a known WNT target gene in the intestinal mucosa and expression of this gene is characteristic for the proliferating crypt compartment [24]. Other genes with decreased expression in the villi were the pro-apoptotic *Bok*, *Htra2*, *Moap1*, *Diablo* and *Bax*. By contrast, as expected, *Cdkn1a* expression was increased in the villi. This gene encodes the cell cycle inhibitor $p21^{cip1/waf1}$ and is induced as a result of decreased levels of the WNT target c-MYC [19]. Other genes with increased expression in the villi were *Noxa, Bid* and *Rambo*.

We subsequently correlated the levels of these 10 putative WNT-regulated genes to the apoptosis-prone phenotype observed in adenomatous intestinal epithelium. This analysis revealed four genes with significantly altered expression in the *Apc* mutant adenomas compared to normal crypts (Fig. 2). *Cdkn1a* expression was found to be upregulated in comparison to normal crypts (2.1-fold, $P < 0.05$; Fig. 2A), while the cell division gene *Birc5* was found to be downregulated (2.8-fold, $P < 0.05$; Fig. 2B). Upregulated pro-apoptotic genes were *Bok* (4.0-fold, $P < 0.001$; Fig. 2C) and *Bax* (1.3-fold, $P < 0.05$; Fig. 2D). The other selected genes did not show this correlation. Our findings demonstrate a shift in the balance between the expression of pro- and anti-apoptotic genes in *Apc* mutant adenomas and suggest a novel link between aberrantly activated WNT signaling and regulation of the pro-apoptotic Bcl-2 family members *Bok* and *Bax* in the intestinal mucosa.

Table 1
Differentially expressed apoptosis-related genes in villi compared with crypts.

Gene symbol(s)	Gene name	Function	Fold change	*P*-value
Birc5/Survivin	Baculoviral IAP repeat-containing 5	Anti-apoptotic	−14.6	9.00E-04
Bok/Mtd	Bcl2-related ovarian killer protein	Pro-apoptotic	−5.2	4.97E-02
Htra2/Prss25	HtrA serine peptidase 2	Pro-apoptotic	−4.7	2.01E-02
Moap1/Map-1	Modulator of apoptosis 1	Pro-apoptotic	−3.9	2.45E-03
Diablo/mac	Diablo homolog (*Drosophila*)	Pro-apoptotic	−2.8	4.99E-03
Bax	Bcl2-associated X protein	Pro-apoptotic	−2.0	1.44E-03
Rambo/Bcl2l13	Bcl2-like 13 (apoptosis facilitator)	Pro-apoptotic	3.3	9.60E-05
Bid	BH3-interacting domain death agonist	Pro-apoptotic	4.9	1.43E-02
Cdkn1a/p21	Cyclin-dependent kinase inhibitor 1	Cell-cycle arrest	6.4	1.65E-03
Noxa/Pmaip1	PMA-induced protein 1	Pro-apoptotic	6.5	4.84E-03

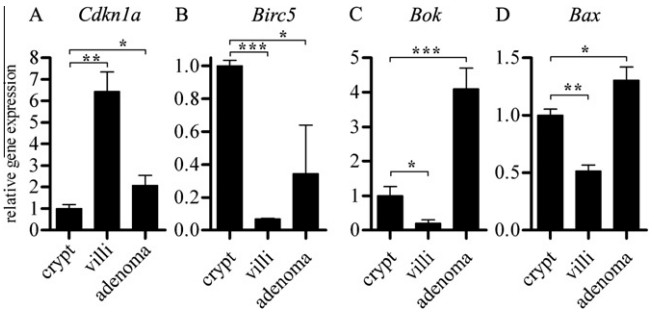

Fig. 2. Selected apoptosis related genes differentially expressed in the intestinal mucosa of $Apc^{Min/+}$ mice. (A–D) RT-MLPA results showing relative gene expression levels for, respectively, *Cdkn1a*, *Birc5*, *Bok* and *Bax* in intestinal crypts, villi and *Apc* mutant adenomas ($n = 4$ per group; $*P < 0.05$; $**P < 0.01$; $***P < 0.001$).

3.3. BOK, BAX and BIRC5 expression in human colonic dysplastic crypts

The above results demonstrate a correlation between loss of APC function and altered expression of apoptosis related genes in mouse intestinal adenomas. In order to establish the expression levels of *BOK, BAX,* and *BIRC5* in the equivalent human disease, especially in the earliest identifiable colonic lesions initiated by aberrantly activated WNT signaling, we set out to analyze the colon mucosa of FAP patients. Pure epithelial cell fractions were isolated from dysplastic crypts, as well as from the base and tables of normal crypts distant from adenomatous tissue, by using laser-aided microdissection (see Supplementary Fig. 1 for details). Gene expression was analyzed by real-time qRT-PCR on cDNA derived from these cells. Expression of *CDKN1A* and the WNT target genes *CD44* [25] and *c-MYC* [26] was studied as control.

Compared to expression in the epithelium at base of human colon crypts, expression levels of *CD44* and *c-MYC* were decreased in the epithelial cells derived from the crypt surface and strongly increased in the dysplastic epithelial cells (Fig. 3A and B), confirming the expected WNT signaling activity in the different cell fractions. In addition, compared to the $Apc^{Min/+}$ mice, we also observed a similar *CDKN1A* expression pattern (Fig. 3C) and, as expected, the *BIRC5* expression levels showed a significant decrease along the crypt-to-surface axis (4.5-fold, $P < 0.001$; Fig. 3D). However, in contrast to the other two established WNT target genes *CD44* and *c-MYC*, *BIRC5* expression was not further increased in the human dysplastic crypts and showed a relatively more variable expression between the individual samples. Notably, this expression pattern was also observed in the $Apc^{Min/+}$ mouse adenomas (Fig. 2B). Examination of *BOK* expression revealed decreased levels at the crypt surface (2.2-fold, $P < 0.05$; Fig. 3E), while expression levels at the colonic crypt base and in dysplastic crypts were similar ($P > 0.05$). *BAX* levels were slightly, but not significantly, decreased at the crypt surface (1.6-fold, $P > 0.05$; Fig. 3F) and, interestingly, almost 2-fold increased in dysplastic crypts ($P < 0.05$). These findings demonstrate that *BOK* and *BAX* are predominantly expressed at the colonic crypt base and in early dysplastic lesions characterized by aberrantly activated WNT signaling.

3.4. APC mutant human colonic dysplastic crypts display increased BAX immunoreactivity

To confirm increased BAX levels in *APC* mutant aberrant crypt foci we studied BAX protein expression in colonic tissue sections derived from the FAP patients by immunohistochemistry. BOK protein expression could not be studied in tissue sections because of

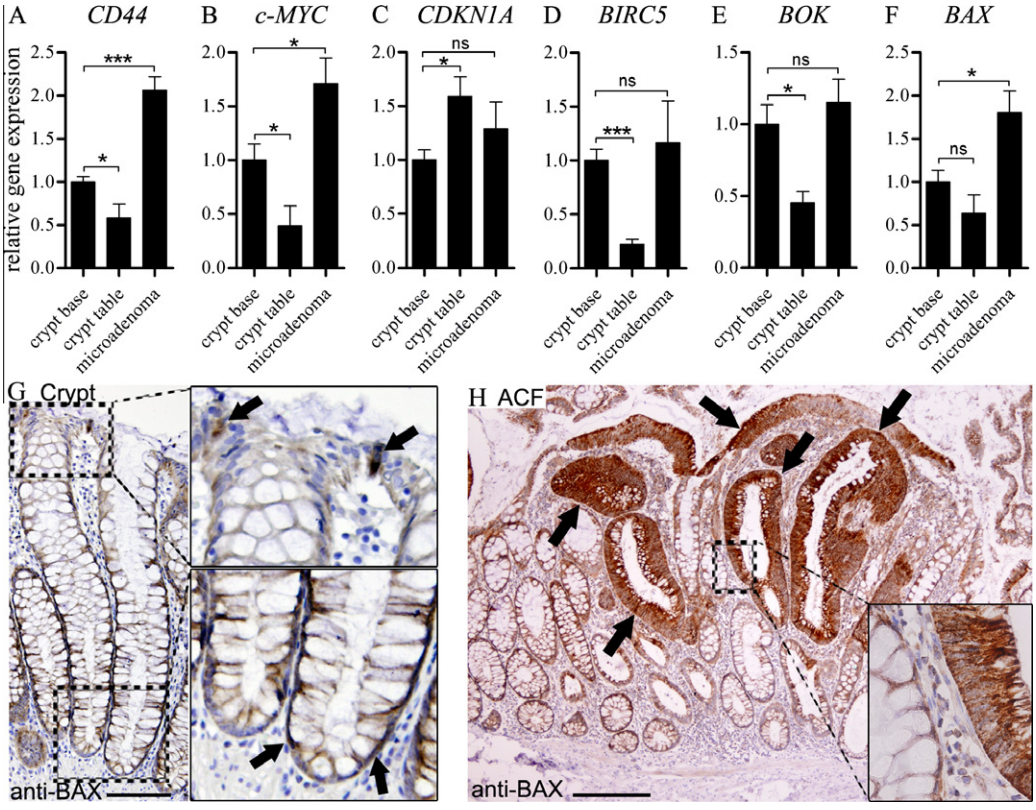

Fig. 3. Pro-apoptotic *BOK* and *BAX* expressions in the intestinal mucosa of FAP patients correlate with β-catenin/TCF target gene expression. (A–F) qRT-PCR results showing relative gene expression levels for, respectively, *CD44*, *c-MYC*, *CDKN1A* ($p21^{CIP1/WAF1}$), *BIRC5*, *BOK* and *BAX* ($n = 4$ per group; $*P < 0.05$; $***P < 0.001$; ns, not significant). (G) BAX immunoreactivity in normal colonic mucosa. Epithelial cells at the intercrypt tables (upper panel) and at the crypt base (lower panel) are positive for BAX immunostaining (arrows; scale bar = 50 μm). (H) Representative picture of a colonic ACF from a FAP patient stained with anti-BAX antibody (arrows; scale bar = 100 μm).

the lack of available high-quality specific antibodies. Histologically normal colorectal mucosa displayed BAX immunostaining in the cytoplasm of epithelial cells at the base of the crypts as well as in epithelial cells higher up the crypt axis and at the crypt tables (Fig. 3G). Compared to normal mucosa, aberrant crypt foci showed strongly increased BAX immunoreactivity (Fig. 3H). These results were consistent with the RT-PCR results and demonstrate high levels of BAX protein in the earliest identifiable neoplastic lesions in human colon.

3.5. WNT signaling controls gene expression of pro-apoptotic BOK and BAX

To test whether gene expression of *BOK* and *BAX* is indeed controlled by β-catenin/TCF-4-mediated signaling we made use of human Ls174T colorectal cancer cells carrying a doxycycline inducible expression plasmid encoding an N-terminally truncated version of TCF-4 [19]. This dominant negative form of TCF-4 is unable to bind β-catenin and strongly inhibits WNT signaling activity. Ls174T cells express mutant β-catenin protein and carry wild-type alleles of *p53* and *APC* [19]. Cells were treated with doxycycline for 24 h and gene expression was analyzed using real-time qRT-PCR. Parental cells expressing the Tet-repressor alone were used as control. As positive control for the effect of transcriptional disruption by dnTCF-4, we measured the relative expression levels of *CD44, c-MYC, CDKN1A* and *BIRC5*. As expected, induction of dnTCF-4 inhibited the expression of CD44, *c-MYC* and *BIRC5*, and strongly induced the expression of *CDKN1A* (Fig. 4A–D). Quantification of *BOK* mRNA levels revealed a reduction of almost 50% after dnTCF-4 induction ($P < 0.001$; Fig. 4E), which was comparable to the reduction of *c-MYC* and *BIRC5* levels. Analysis of *BAX* gene expression revealed a similar down-regulation ($P < 0.01$; Fig. 4F). These results imply that expression of pro-apoptotic *BOK* and *BAK* in the intestinal epithelium is controlled by β-catenin/TCF-4 mediated transcription.

4. Discussion

Dysregulated WNT signaling, caused by loss of functional APC is the driving force behind the initiation of dysplastic aberrant crypts in FAP patients and $Apc^{Min/+}$ mice and has been observed in the majority of sporadic colorectal tumors [5,6]. In the current study, we demonstrate that loss of APC function is associated with a strong initial increase in the rate of epithelial cell apoptosis in the adenomatous lesions of $Apc^{Min/+}$ mice. This finding is in line with previous reports showing increased apoptosis in the intestinal mucosa after conditional deletion of *Apc* [9,10]. The sharp increase in apoptosis supports a relation between WNT signaling and the expression of apoptosis-related genes in the intestinal mucosa. Indeed, gene expression analysis along the normal crypt-villus axis and in intestinal adenomas of $Apc^{Min/+}$ mice revealed the differential expression of two pro-apoptotic Bcl-2 family members, *Bok* and *Bax* (Fig. 2). BAX is the prototypic apoptosis-promoting member of the Bcl-2 family and is directly involved in the execution of apoptosis by inducing the release of mitochondrial cytochrome c [27]. Interestingly, inactivating *BAX* mutations have been found in over 50% of human microsatellite instable colon tumors, suggesting a suppressor role for the wild-type *BAX* gene in a p53-independent pathway for colorectal tumorigenesis [28]. Similar to BAX, BOK contains the conserved Bcl-2 homology (BH) domains 1–3 and, likewise, exerts its pro-apoptotic function through the mitochondrial pathway [29,30]. *Bok* expression was previously demonstrated in the basal crypt compartment of mice and, importantly, increased expression was associated with increased epithelial apoptosis at this position [15]. We demonstrate herein that *BOK* and *BAX* are prominently expressed in the human colonic basal crypt compartment as well as in the dysplastic crypts of FAP patients. Furthermore, the expression of *BAX* was almost 2-fold increased in these dysplastic lesions (Fig. 3F), which was confirmed by immunohistochemistry (Fig. 3H). These results demonstrate that increased *BOK* and *BAX* expression is an early event in

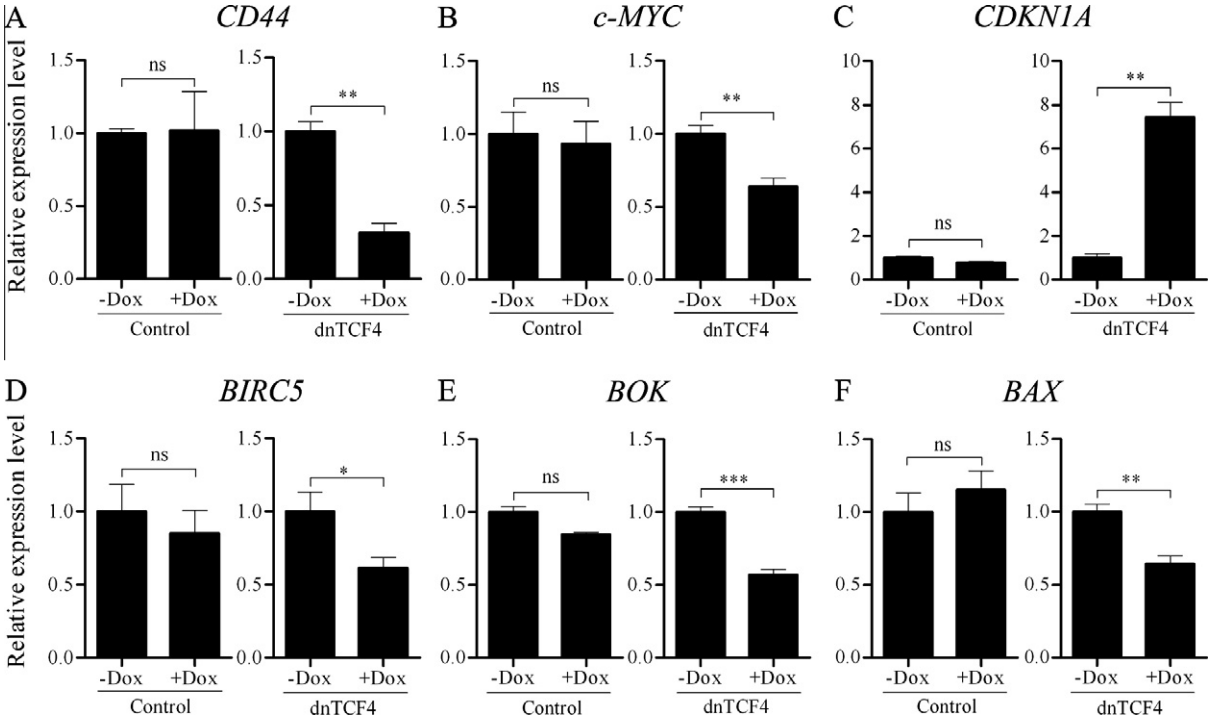

Fig. 4. WNT signaling regulates gene expression of pro-apoptotic *BOK* and *BAX* in human epithelial colon cells. Relative gene expression levels measured by qRT-PCR in Ls174T cells carrying doxycycline-inducible TCF-4 or control cells. Cells were incubated with or without doxycycline (Dox) for 24 h. (A and B) Relative expression levels of the WNT target genes *CD44* and *c-MYC*. C, *CDKN1A* ($p21^{CIP1/WAF1}$). (D) *BIRC5*. (E and F) *BOK* and *BAX* ($*P < 0.05$; $**P < 0.01$; $***P < 0.001$; ns, not significant).

intestinal tumorigenesis and suggest that their expression is dependent on WNT signaling. Indeed, experiments employing human colorectal cancer cells carrying inducible dnTCF-4 constructs corroborated the involvement of β-catenin/TCF-4-mediated signaling in the regulation of *BOK* and *BAX* (Fig. 4). Relevant to these findings is the recent identification of c-MYC as a key downstream mediator of WNT regulated gene expression following APC loss [10,31]. Deregulated c-MYC expression is associated with an increased sensitivity to apoptosis [32], and it has been reported that *BAX* is a c-MYC target gene [33]. Further studies are needed to elucidate the role for c-MYC in the regulation of apoptotic signaling in the context of colorectal cancer.

Of interest, although we and other groups have demonstrated that *BIRC5* is a WNT target gene in intestinal epithelial cells [19,24], *BIRC5* expression levels were not further increased in the mouse and human adenomatous lesions. Moreover, expression profiling of normal mucosa showed that *BIRC5* expression was highly restricted to the proliferating crypt compartment (Figs. 2B and 3 D). These results are in agreement with earlier studies demonstrating that BIRC5/Survivin primarily functions as a mitotic regulator [23], and suggest an essential role for this protein in the proliferation of intestinal epithelial cells. It should be noted that the dynamics of apoptosis in conditions of enhanced proliferation depends on many factors: pro-apoptotic BOK and BAX can interact with anti-apoptotic Bcl-2 family members, such as, BCL-2, BCL-xL, BCL-W and MCL-1 [29,34], and the balance between all these different pro- and anti-apoptotic components defines the threshold of responsiveness to apoptotic signals. This decision making process is further influenced by the inhibitor of apoptosis (IAP) family of proteins [35]. The increased apoptosis observed in intestinal epithelial cells lacking APC demonstrates a functional role of WNT signaling in shifting this balance, however, whether this indeed affects adenoma formation and progression needs further exploration.

Taken together, our findings demonstrate a novel link between dysregulated WNT signaling and the transcription of pro-apoptotic Bcl-2 family members at the early stages of WNT-driven intestinal tumorigenesis. Accordingly, exploitation of this relationship might prove useful in the prevention and treatment of intestinal cancer.

Acknowledgments

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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.2010.12.070.

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