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CD24 Induces the Activation of β-Catenin in Intestinal Tumorigenesis

Fokra Ahmad[1,4], Kazanov Dina[1], Bedny Faina[2], Brazowski Eli[2,4], Varol Chen[3,4], Kraus Sarah[1,4], Arber Nadir[1,4]* and Shapira Shiran[1,4]

[1]*The Integrated Cancer Prevention Center, Tel Aviv University, Tel Aviv, Israel*
[2]*Pathology Institute, Tel Aviv University, Tel Aviv, Israel*
[3]*Research center of Digestive Tract and Liver Diseases, Tel Aviv Sourasky Medical Center, Tel Aviv, Israel*
[4]*Sackler School of Medicine, Tel Aviv University, Tel Aviv, Israel*

Abstract

Background: CD24 is a glycosylphosphatidylinositol-linked protein that functions as an adhesion molecule and is overexpressed at an early stage of CRC. The Wnt/β-catenin signaling pathway plays an important role in the CRC carcinogenesis process. We have previously shown that CD24 could affect the tumorigenesis process in *Apc*$^{Min/+}$ mice.

Methods: CD24-inducible 293T-REx™ cells previously developed in our lab, HT29 and SW480 CRC cells were used to study this interaction *in vitro*. *Apc*$^{Min/+}$ and CD24 knockout (KO) mice, both on a C57BL/6J genetic background, were crossed to generate double KO transgenic mice. Large bowel polyps were counted macroscopically and small bowel polyps were verified microscopically.

Results: *In vitro* Western blotting analyses showed that induced expression of CD24 led to the activation of β-catenin. Co-immunoprecipitation studies of CD24 and β-catenin indicated that these two proteins might be interacting. Cytoplasmic/nuclear fractionation showed that more active β-catenin enters the nucleus in cells that express CD24 compared to control cells. In addition, TOP/FOP luciferase reporter assay showed a significant increase in luciferase activity upon CD24 expression induction.Conversely, down-regulation of CD24 by mAbs and siRNA caused a decrease in the levels of active β-catenin.

Depletion of CD24 alleles in *Apc*$^{Min/+}$ mice led to a significant reduction in the number of polyps in the small and large intestine. The *Apc*$^{Min/+}$mice displayed severe splenomegaly compared to *Apc*$^{Min/+}$/*CD24*$^{+/-}$ mice and double KO mice were similar to WT mice with normal spleen size. HGB and RBC levels were significantly lower than in the double KO mice.

Conclusions: CD24 plays a major role in intestinal tumorigenesis. It interacts with the Wnt pathway by activating β-catenin. Down regulation of CD24 reduces the polyp burden in mice and therefore it might serve as an important target in the therapy of CRC.

Keywords: Colorectal cancer; β-catenin; CD24; Apc-min

Introduction

Colorectal cancer (CRC) is one of the major health concerns worldwide; it is the second leading cause of cancer death. The sequence of the molecular events that occur during the initiation and progression of CRC have been identified during the last two decades [1-4]. However, the exact mechanisms by which these alterations are involved in this process are still not fully understood. The earliest mutations that have been identified in this sequence involve components of the Wnt/β-catenin signaling pathway; β-catenin/TCF-dependent transcription factor is responsible for the regulation of expression of several oncogenes including, cyclin D1 and c-myc [5-9]. Previous results from our lab, including immunohistochemical staining of 398 human samples at different stages, have shown that CD24 is overexpressed in adenomas and adenocarcinomas of the gastrointestinal (GI) tract. Moreover, in an experimental model of chemically induced inflammation-associated CRC, CD24 knockout (KO) mice were resistant [2,10].

CD24, also known as heat-stable antigen (HSA), is a short mucin-like glycosyl-phosphatidyl-inositol (GPI)-linked protein, with a molecular weight ranging from 38 to 70 kDa [10,11]. It contains a small peptide of 31 amino-acids that is homologous to the mouse CD24a [12,13]. CD24 is expressed on the outer surface of the cytoplasmic membrane, but it has no trans-membrane domain. It functions as an adhesion molecule and has a potential role in signal transduction [10,14].

Expression was observed mainly in B-lymphocytes, but was also observed in the developing brain and to some extent on various epithelial cells [15-18].

An increasing number of studies have reported high levels of CD24 expression in most/all human malignancies, where it is usually associated with poor prognosis. Numerous immuno-histochemical and expression array studies have detected overexpression of CD24 in B-cell lymphomas, gliomas, and many carcinomas, including: small-cell and non-small-cell lung, hepatocellular, uterine, ovarian, breast, prostate and pancreatic [19-22]. In many of these studies CD24 is linked to a metastatic phenotype, which is mostly mediated by selectins, particularly P-selectin which is believed to be its major ligand. For over a decade, our laboratory has focused on studying the role of CD24 in the multi-step process of GI carcinogenesis. Sagiv et al. have shown

***Corresponding author:** Nadir A, Professor of Medicine and Gastroenterology, Yechiel and Helen Lieber Professor for Cancer Research, Head of the Integrated Cancer Prevention Center, 3/3 Floor, Arison Medical Tower, Tel Aviv Sourasky Medical Center, 6th Weizmann St. Tel Aviv 64239, Israel, Tel: +972-3-6974968, +972-52-4266595; Fax: +972-3-6974867; E-mail: nadira@tlvmc.gov.il, narber@post.tau.ac.il

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that CD24 is overexpressed at an early stage of CRC carcinogenesis process [2]. We later showed that CD24 downregulation using siRNA or anti-CD24 monoclonal antibodies in CRC and pancreatic cancer cell lines caused a significant decrease in survival and proliferation [1,2]. In breast cancer, it has been shown that β-catenin down-regulates the expression of CD24 [23] which suggests the existence of an interplay between CD24 and the Wnt pathway. However, unlike CRC, the reports about the role of CD24 in breast cancer are contradictory [23-25].

The Wnt/β-catenin signaling pathway is a key regulator in numerous cellular processes including stem cell maintenance, fate decision, and cell cycle control. β-catenin, the main denominator of this pathway, has several cellular functions. At the cell membrane, it is associated with E-cadherin and participates in the formation of the adherent junctions. In the cytoplasm, β-catenin can form complexes with a multitude of proteins, including the β-catenin destruction complex consisting of APC, AXIN2, GSK3β and CK1α [26]. In normal epithelial tissue, APC induces the degradation of β-catenin [27,28]. Opposing this, under physiological conditions, the Wnt ligand binds to the Wnt receptor impeding the β-catenin destruction [29]. When β-catenin accumulates in the cytoplasm it translocate into the nucleus where it associates with DNA-binding proteins of the Tcf family of transcription factors (Tcf1, Lef, Tcf3 and Tcf4) activating several early response target genes such as Jun, Myc and cyclin D1 [29-31].

The $Apc^{Min/+}$ mouse model is an excellent system to study intestinal tumorigenesis. it contains a truncating mutation at codon 850 of the Apc gene; a mutation that was proven to be important for sporadic CRC tumorgenesis as well. Homozygosity for mutant Apc is embryonically lethal [32]. The C57BL/6J background mice that are $Apc^{Min/+}$, are considered to be predisposed to multiple intestinal neoplasms (Min). They develop approximately 30 small intestinal polyps and a few colonic adenomas that rarely progress to invasive adenocarcinoma [32,33]. The main goal of this study is to better understand the mechanisms by which CD24 is involved in the multi-step process of intestinal tumorigenesis. So far, only a limited number of studies have tried to investigate the mechanisms by which CD24 contributes to tumor progression. Therefore, it is essential to investigate CD24 protein-protein interactions in CRC. Our main focus will be in trying to elucidate the interplay between CD24 and the Wnt signaling pathway. We have previously shown in this mouse model that even breeding heterozygous CD24 mice drastically prevents adenoma formation [34].

Herein we confirm these results by establishing a new colony of double KO mice and elucidate how CD24 affect the β-catenin pathway.

Methods

Cell cultures

Human CRC (SW480, HT-29) and 293T-REx™ cell lines were obtained from the American Type Culture Collection (Manassas, VA), cultured in DMEM supplemented with 5% (v/v) heat-inactivated (HI) Fetal bovine serum (FBS), 1% glutamine, 1% penicillin and streptomycin at 37°C, in an atmosphere of 95% oxygen and 5% CO_2. CD24-inducible 293T-REx™ system is a tetracycline-regulated mammalian expression system. pcDNA4/TO-CD24 was transfected into 293T-REx™ stable cells expressing the tetracycline repressor from the pcDNA6/TR vector (Invitrogen), using the calcium phosphate transfection method [35], HT29 clone E, stably transfected with two small interfering RNA (siRNA) with sequences according to the cDNA of CD24 [1], and SW480 clones 1, Stable cell lines expressing CD24 were generated by transfecting the full-length human CD24 coding region; SW480 clone 4, cells stably transfected with the control vector [34] were developed in our lab.

Calcium phosphate transfection

Cells were seeded for 50-70% confluence one day prior to transfection. For a 10-cm plate, a mixture containing 20 μg DNA of GFP-β-catenin construct (a kind gift from Prof. Rina Arbersfeld, Faculty of Medicine, Tel Aviv University) [36] and 50 μl of 2.5M $CaCl_2$ in sterile TEx1 solution at a final volume of 500 μl, is added dropwise to 500 μl of 2×Hepes buffered saline (HeBS) while bubbling the HeBS. The mixture was then added to the cells. 12-16 hours after transfection the medium was replaced. For optimal protein expression the cells were assayed 48 hours after transfection.

Protein techniques

Western blot analysis: Exponentially growing cells were collected and washed in ice-cold phosphate buffered saline (PBS). Cell pellets were resuspended in lysis buffer (50 mM Tris-HCI (pH 7.5), 5 mM EDTA, 100 mM NaCl, 1% Triton and 50 mM NaF) supplemented with a protease inhibitor cocktail. For Western blotting, samples containing 50 μg of total cell lysate were loaded onto 12% SDS-polyacrylamide gels and subjected to electrophoresis. Proteins were transferred to Hybond-C membranes (Amersham, Arlington Heights, IL) in transfer buffer (25 mM Tris, 190 mM glycine, and 20% methanol), using a Trans Blot transfer apparatus at 300 mA for 1 hour at room temperature (RT). Membranes were blocked with blocking buffer (5% skim milk in PBS containing 0.05% Tween-20 (PBS-T)) for 1 h. The membranes were incubated with a diluted primary antibody for 1 h at RT. Membranes were then washed three times for 10 min in PBS-T, incubated with horseradish peroxidase (HRP)-conjugated secondary antibodies (1:2000-10,000) for 1 hour, thoroughly washed again and immune detection was performed using enhanced chemiluminescence (ECL).

Primary antibodies: mouse anti-CD24 mAb (SWA11: a generous gift from Prof. Peter Altevogt, German Cancer Reseach Center, and Prof. Rolf Stahel, University of Zurich, Switzerland), polycolonal anti-actin(Santa Cruz Biotechnology), monoclonal anti-tubulin (Sigma-Aldrich), anti-active-β-catenin mAb (clone 8E7), was purchased from Millipore, (Billerica, MA). Anti-β-catenin (H-102) was purchased from Santa Cruz Biotechnology (Santa Cruz, CA).

Immunoprecipitation: Cells were lysed in lysis buffer and centrifuged to remove insoluble material. After preclearing with protein A/G-agarose, 300 μg of protein lysates will be immunoprecipitated with 20-40 μg of the anti-CD24 antibody together with protein A/G agarose. Precipitates were washed thrice with PBS, boiled in reducing SDS-PAGE sample buffer, subjected to 10% SDS-PAGE, and then Western blotted. The blot was incubated with anti (active/total)-β-catenin antibody, the IP was repeated by first immunoprecipitating β-catenin (or other Wnt components) and then Western blotting CD24.

Luciferase reporter activity assay: Trasfections were performed by standard calcium phosphate transfection or other reagents according to standard protocols. Cells were seeded in 6-well plates, and the next day, 60-70% confluent dishes, were co-transfected with 1 μg of DNA of the super TOPFLASH or the control super FOPFLASH plasmid. 0.1 μg of β-galactosidase or Renila luciferase plasmid was used to evaluate the transfection efficiency (all plasmids are a generous gift from Prof. Levana Sherman, Faculty of Medicine, Tel Aviv University). The Wnt pathway was activated by transfection of a β-catenin encoding plasmid and treating with 30 mM LiCl for 24 h.

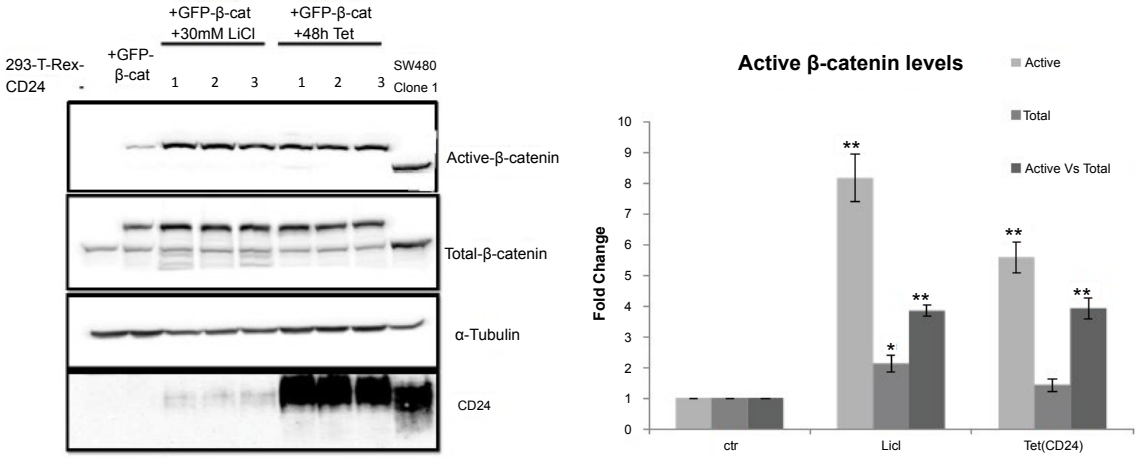

Figure 1: High expression of CD24 activates β-catenin
GFP-β-catenin was transfected into 293T-REx-CD24 cells and analyzed by Western blotting with anti-active/total β-catenin and anti-CD24. These cells were treated with 30 mM Tet for either 24 or 48 hours. SW480-clone 1 cells overexpressing CD24 were used as a positive control. Active and total β-catenin levels upon treatment with 30 mM of lithium chloride (LiCl) or Tet. Results shown are representative of two independent experiments. This shows that following the Tet induction of CD24 expression in 293T-REx-CD24 cells, active- β-catenin levels were significantly increased. LiCl, a well-known activator of β-catenin, was used as a positive control and induced a ~10-fold increase in active- β-catenin and a ~2-fold increase in total- β-catenin. CD24 induction by Tet treatment showed a ~3.5-fold increase in active-β-catenin but no significant change in total- β-catenin. Tubulin was used as a loading control and in the analyses of both proteins for normalization. In addition, active-β-catenin levels were normalized to the total- β-catenin, which showed a ~4.5-fold increase upon LiCl treatment and a ~2.5-fold increase upon CD24 induction by Tet. CD24 expression was also validated. Error bars represent ± SD. **P*<0.05, ***P*<0.005.

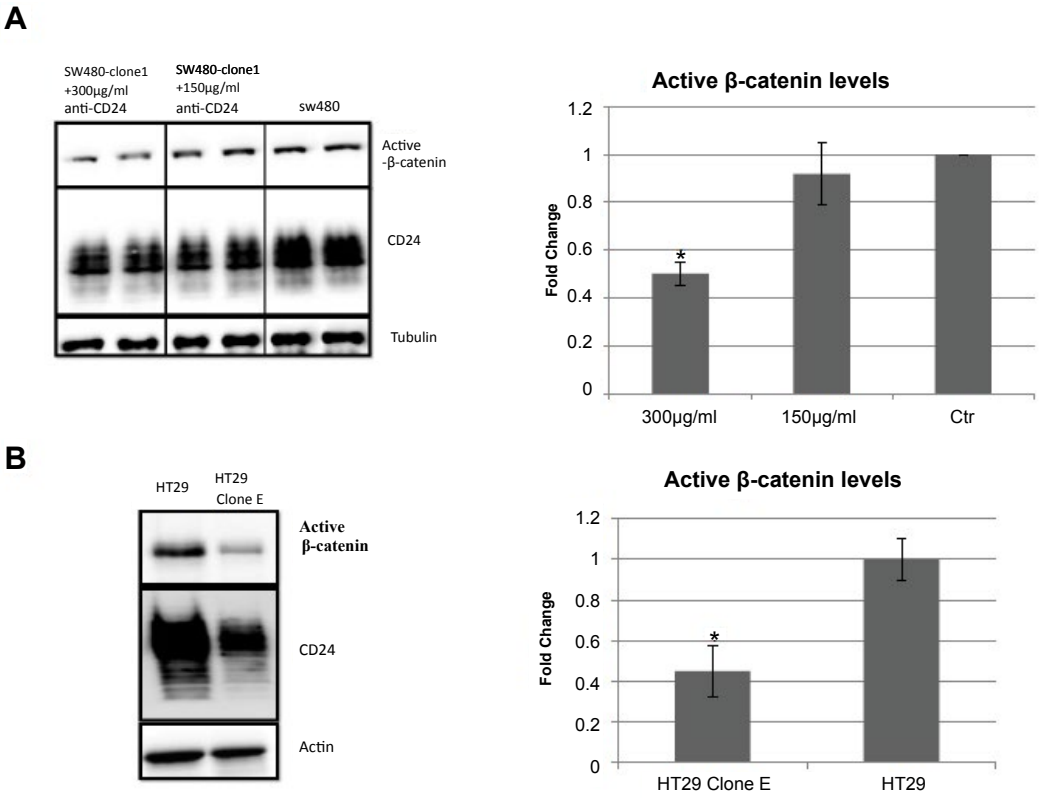

Figure 2: Down-regulation of CD24 deactivates β-catenin
SW480-clone 1 cells (cells transfected with CD24) compared to SW480-clone 4 cells (cells transfected with empty vector). Cells were grown on 6-well plates and treated with 150 or 300 (µg/ml) of humanized anti-CD24 antibody for 72 hours. Duplicates of each concentration (150/300) and control (without treatment) were analyzed. The samples were examined by western blotting analysis and the expressions of CD24, tubulin and active β-catenin were detected. Western blotting results confirmed that the anti-CD24 antibody treatment succeeds in down regulating the protein levels in the SW480-clone1 cells, and more importantly, it induces a reduction in the levels of active-β-catenin. Cells which were treated with 300 (µg/ml) of humanized anti-CD24 antibody for 72 hours showed more than 50% reduction in active-β-catenin levels (Figure 4, left), whereas treating SW480 cells that do not express CD24 with the same antibody did not affect the levels of active-β-catenin(A). In HT29 clone E cells, CD24 is down regulated by the use of siRNA methods, the results, demonstrated a more than two fold decrease in the levels of active-β-catenin (B). Error bars represent ±s.d. **P*<0.05.

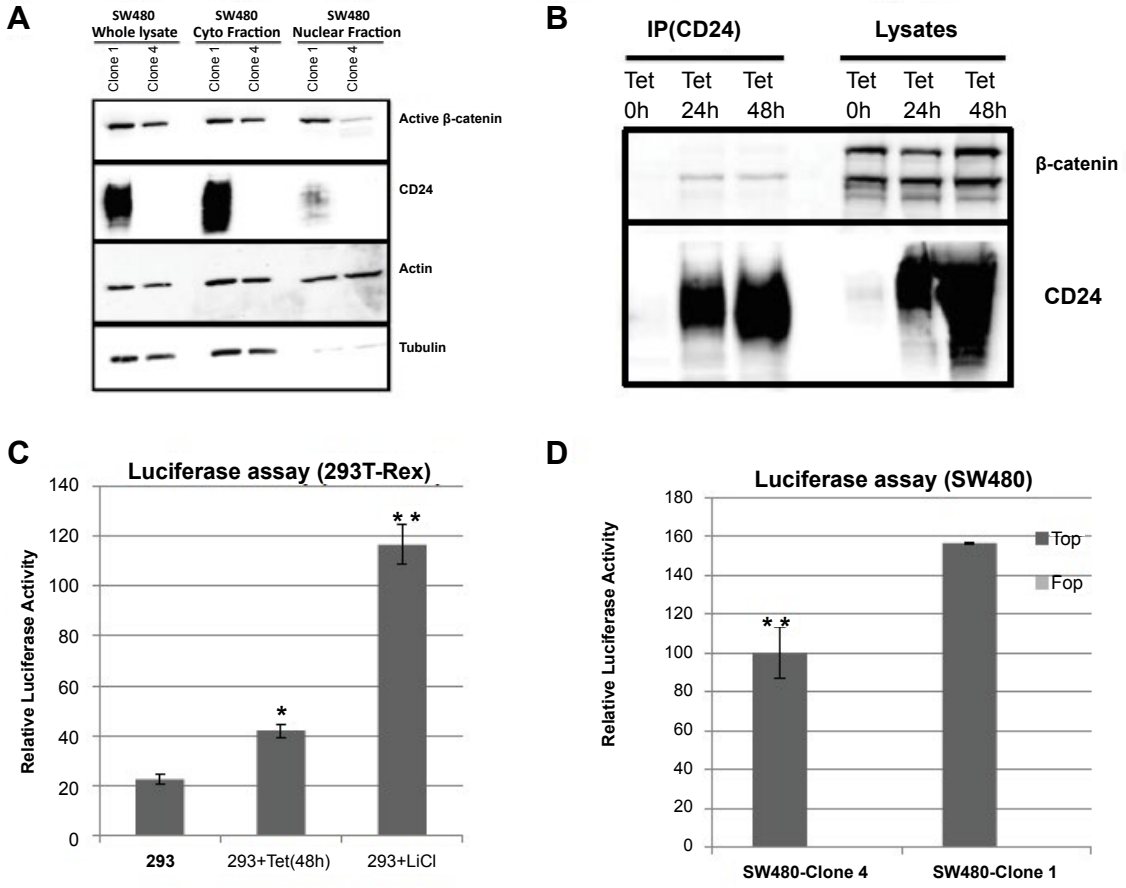

Figure 3: CD24 interacts with β-catenin and induces its translocation into the nucleus
Cytoplasmic/nuclear fractioning performed on SW480 cells shows that in clone 1, there were higher levels of β-catenin compared to clone 4 (A). IP was performed in 293T-REx™ cells to isolate the CD24 complex. Then this complex was immunoblotted against β-catenin, and it shows that β-catenin one of the proteins in the CD24 complex (B), 293T-REx™, and SW480 cells were transfected with 1 μg of DNA of the super TOPFLASH or the control super FOPFLASH plasmid. 0.1 μg of β-galactosidase or Renila luciferase plasmid were used to evaluate the transfection efficiency. In both cell lines CD24 induces an increase in the luciferase activity. The Wnt pathway activator LiCl was used as a positive control (C,D). Error bars represent ± SD. *P<0.05, **P<0.005.

The luciferase levels were measured using the luciferase assay system (Promega), and normalized to the β-galactosidase or Renila luciferase activity [37].

CD24 and Apc$^{Min/+}$ double knockout mice: Apc$^{Min/+}$ mice used in this study are a kind gift from the lab of Prof. Yinon Ben-Neriah (Faculty of Medicine, The Hebrew University of Jerusalem). These mice were first mated with CD24$^{-/-}$ which were kindly provided by Prof. Peter Altevogt (German Cancer Research Center, Heidelberg, Germany). The Apc$^{Min/+}$ and the CD24$^{-/-}$ mice were mated to produce Apc$^{Min/+}$/CD24$^{+/-}$ pups which were then mated again with CD24$^{-/-}$ again to reverse the genotype of CD24$^{+/-}$ to CD24$^{-/-}$. Genomic DNA was extracted from mouse tails, and the genotype of Apc$^{Min/+}$ was verified by PCR. Three primers were used to identify the APC genotype (Table 1). For the CD24 genotype, two PCRs were conducted in order to detect the Neomycin cassette which replaces the CD24 Exon1 in two alleles of CD24$^{-/-}$ genotype and in one allele of CD24$^{+/-}$ (Table 1).

Visualization, counting and histochemistry of polyps: Polyps were scored in small and large intestine by using two different methods. Upon sacrifice, the gut was removed, flushed with PBS, filled with 4% buffered formaldehyde and opened longitudinally. After overnight fixation, the sections were stained with hematoxylin/eosin at the Pathology department, Tel Aviv Medical Center. These slides were then visualized under light microscope and the number of polyps was counted. For the large intestine, Colonic polyps were counted using mice colonoscopy prior to sacrifice which was performed by a highly skilled technician. In addition, blood counts and spleen size of these mice were measured in order to analyze the effects on the expected intestinal bleeding.

Sequence 5' --> 3'	Primer Type
GCCTTATGCCAAAGTGGAA	APC-Reverse
TTCTGAGAAAGACAGAAGTTA	APC-MUT Forward
GCCATCCCTTCACGTTAG	APC-WT Forward
TTGAACAAGATGGATTGCACGCA	Neo-cassette-Forward
TGATCGACAAGACCGGCTTCC	Neo-cassette-Reverse
TAGCAGATCTCCACTTCCG	Exo1-Forward
GTAGGAGCAGTGCCAGAAGC	Exo1-Reverse

Table 1: Primers used for genotyping.

Statistical Analysis: Data from the *in vitro* studies are presented as mean±SD of sets of data as determined in triplicates. Statistical significance between treatments was determined by Student t-test, P values <0.05 were considered significant.

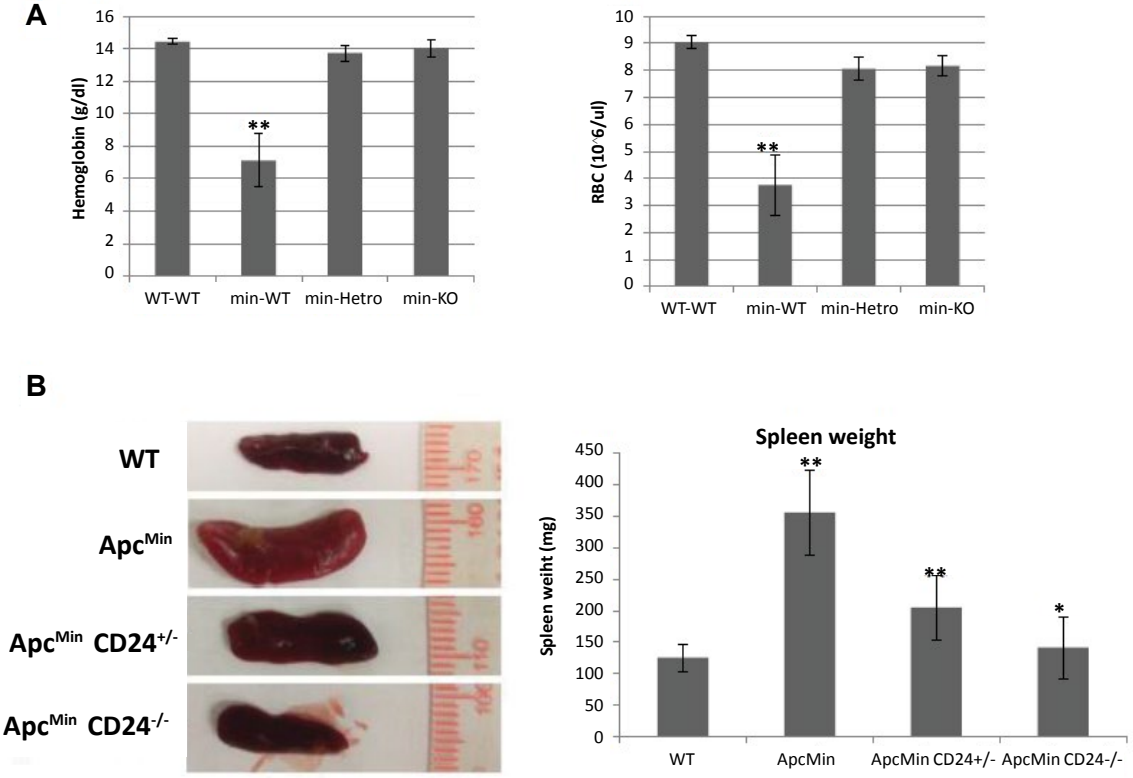

Figure 4: CD24 depletion in Apc$^{Min/+}$ mice prevents anemia and splenomegaly
At the age of 24 weeks, before sacrifice, blood tests were performed on the mice. Results showed a decrease in RBC and HGB levels for the Apc$^{Min/+}$ mice compared to WT mice, or mice with the depletion of one allele of CD24 in the Apc$^{Min/+}$ mice (min-Hetro) or complete KO of CD24 (min-KO) (A). Spleen weight was measured following sacrifice and shows that Apc$^{Min/+}$ mice developed splenomegaly, while min-Hetro, and min-KO mice had normal spleen size (B). Error bars represent ± SD. *P<0.05, **P<0.005.

Results

High expression of CD24 activates β-catenin

293T-REx-CD24 cells transfected with GFP-β-catenin, were treated with 30 mM Tetracycline (Tet) for either 24 or 48 hours to induce CD24 expression. SW480-clone 1 cells overexpressing CD24 were used as a positive control. Active and total β-catenin levels were examined upon treatment with 30 mM of lithium chloride (LiCl), a well-known activator of β-catenin, or Tet. This experiment mainly showed that following the Tet induction of CD24 expression in 293T-Rex-CD24 cells, active- β-catenin levels were significantly increased. LiCl was used as a positive control and induced a ~10-fold increase in active-β-catenin and a ~ 2-fold increase in total- β-catenin. CD24 induction by Tet treatment showed a ~3.5-fold increase in active- β-catenin but no significant change in total- β-catenin. In addition, active- β-catenin levels were normalized to the total- β-catenin which showed a ~4.5-fold increase upon LiCl treatment and a ~2.5-fold increase upon CD24 induction by Tet. CD24 expression was also validated (Figure 1).

These results raised the question whether CD24 is solely responsible for the increase in active-β-catenin levels or that maybe the Tet itself has an effect on the activation of the protein. To answer this question, 293T-REx™ cells (supplementary figures) were treated with Tet for 48 hours and compared to untreated cells. No increase in the levels of active/total β-catenin were found, suggesting that the increase in active-β-catenin levels, which was observed previously, is indeed CD24-dependent and not related to the procedure of Tet treatment itself.

Down-regulation of CD24 deactivates β-catenin

To further establish the association between CD24 levels and the activation of β-catenin, we aimed to study the effects of down-regulation of CD24 on the level of active-β-catenin in SW480-clone 1 cells (stable transfected cells expressing CD24) and compare it to SW480-clone 4 cells (stable transfected cells with empty vector). The Western blotting confirmed that the anti-CD24 antibody treatment succeeded in down regulating the CD24 levels in the SW480-clone1 cells, and more importantly, this induced a reduction in the levels of active-β-catenin. Cells which were treated with 300 μg/ml of humanized anti-CD24 antibody (developed by us) for 72 hours showed more than 50% reduction in active-β-catenin levels (A), whereas treating SW480 cells that do not express CD24 with the same antibody did not affect the levels of active-β-catenin (supplementary figures). In addition, in HT29 clone E, [1], down-regulation of CD24, by sihRNA, resulted in more than a twofold decrease in the levels of active-β-catenin (Figure 2B).

CD24 interacts with β-catenin and induces its translocation into the nucleus

CD24 influence on the levels of active β-catenin is assumed to be through stabilizing and relocating it to the nucleus where it affects the expression of several genes.

To examine this, we have performed excytoplasmic/nuclear fractionation which showed that more active β-catenin enters the nucleus in SW480 cells that express CD24 (clone1) compared to control cells (clone 4), and that the increased levels of active β-catenin

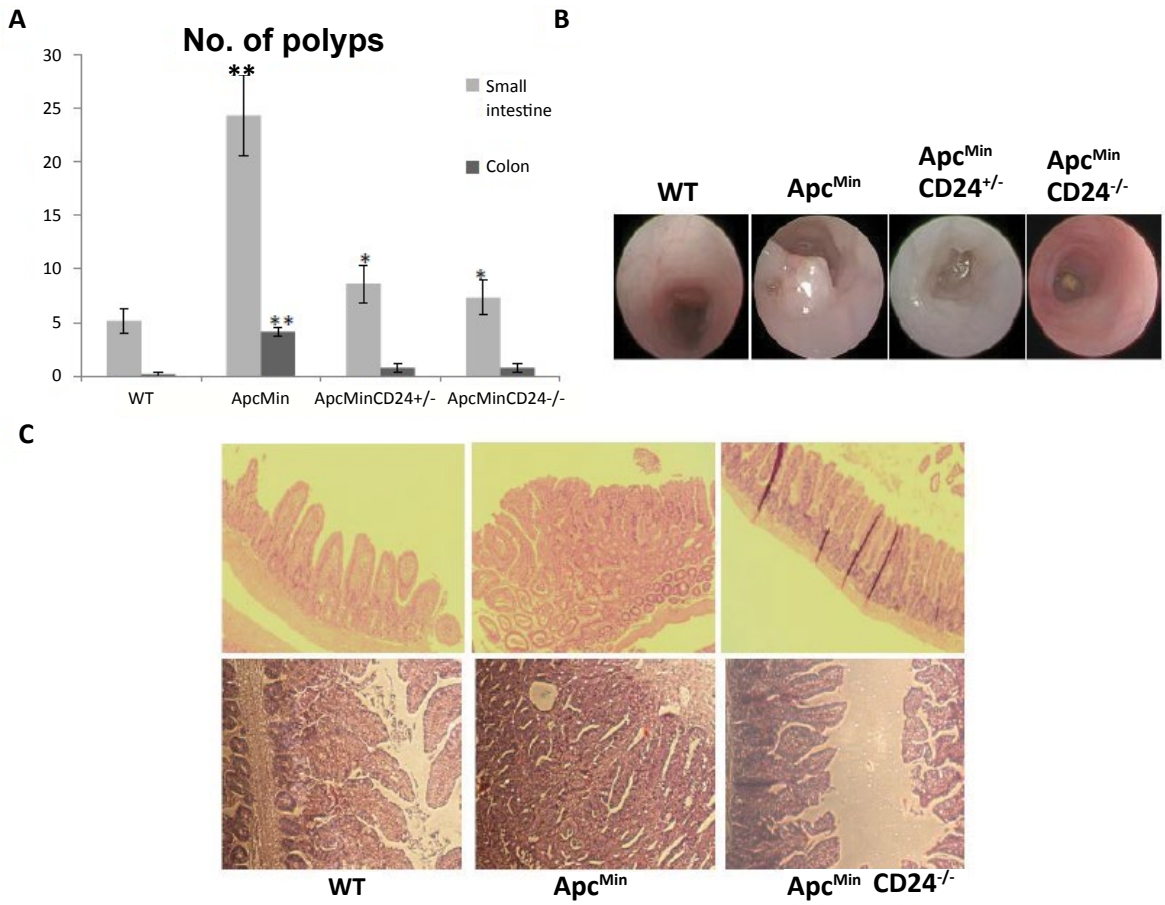

Figure 5: CD24 depletion in Apc$^{Min/+}$ mice reduce the number of intestinal polyps
Apc$^{Min/+}$ mice (n=14) developed ~24.3 ± 3.7 adenomas and several carcinomas in the small intestine by the age of 24 weeks. The *Apc^{Min}/CD24$^{+/-}$* mice (n=10) developed 8±1.4 polyps and the *Apc^{Min}/CD24$^{-/-}$* (double KO) (n=12) mice developed ~7 ± 1.7 polyps (A,B). Colonoscopy showed a significant reduction in the number and size of polyps upon depletion of CD24 alleles (A,C). Error bars represent ± SD. **P*<0.05, ***P*<0.005.

is particularly evident in the nuclear fraction. In addition, the changes in β-catenin/TCF association, which ultimately induces the expression of the target gene were examined by using the β-catenin driven TOP/FOP luciferase reporter assay. There was a significant increase in Luciferase activity upon induction of CD24 expression in both SW480 and 293T-REx^{TM} cells.

Additionally, IP assay was performed to detect the interaction of CD24 with components of the Wnt pathway. Pulling down of CD24 complex does show that it interacts with β-catenin. However, we have failed to show, by this method, that this complex contains other Wnt pathway components (data not shown).

CD24 depletion in Apc$^{Min/+}$ mice prevents anemia and splenomegaly

It is known that Apc$^{Min/+}$ mice develop anemia as a result of the intestinal (polyp) bleeding, and splenomegaly (due to extra-medulary hematopoiesis), the former of which could be detected in a blood test. However, blood counts of the mice at 14 weeks did not show a significant difference in the hemoglobin or RBC levels between Apc$^{Min/+}$ mice, mice that were WT, or mice that were CD24 KO (supplementary figure). However, at the age of 24 weeks, Apc$^{Min/+}$ mice presented with anemia, and the blood count showed a serious decrease in the levels of hemoglobin or RBCs compared to the WT and CD24 double KO mice.

RBC level was 3.8 ± 2.5 in the Apc$^{Min/+}$ significantly lower than in the double KO mice (8.2 ± 0.9) and the WT (9 ± 0.4) (p=0.0009). In the current study the Apc$^{Min/+}$ displayed severe splenomegaly (355 ± 68 mg) compared to (205 ± 51 mg) in Apc$^{Min/+}$/CD24$^{+/-}$ mice and (141 ± 49 mg) in double KO mice similar to WT mice (p=0.006).

CD24 depletion in Apc$^{Min/+}$ mice reduces the number of intestinal polyps

Apc$^{Min/+}$ mice are considered to be predisposed to multiple intestinal neoplasms (Min). They develop approximately 30 small intestinal polyps, which rarely progress to invasive adenocarcenoma. In order to investigate the role of CD24 in intestinal tumorigenesis, we have decided to examine the effects of knocking out CD24 on the developing intestinal polyps in these mice. Here we show that depletion of CD24 alleles in Apc$^{Min/+}$ mice led to a significant reduction in the number of polyps in the small and large intestine (Figures 3 and 4). C57BL6/J mice carrying the ApcMin$^{/+}$ mutation developed ~24.3 ± 3.7 adenomas in the small intestine by the age of 24 weeks. The Apc$^{Min/+}$/CD24$^{+/-}$ mice developed 8 ± 1.4 polyps, and the Apc$^{Min/+}$/CD24$^{-/-}$ (double KO) mice developed only ~7 ± 1.7 polyps (p=0.006) (Figure 5).

Discussion

CD24 plays an important role in the multi-step process of CRC

carcinogenesis. Here, we provide further evidence to support this role of CD24. It is only in the last few years that the role of CD24 is being elucidated [25,38-40].

Here we show that CD24 induces the activation of β-catenin. Inducing the expression of CD24, using the293T-REx™ inducible system, caused a significant increase in the level of active-β-catenin, but not that of total-β-catenin. In contrast, down-regulating CD24 expression in SW480 and HT29 CRC cell lines resulted in a more than two fold decrease in the levels of active-β-catenin. These findings suggest that CD24 does not affect the expression of β-catenin and does not increase its protein level, but affects the activation of β-catenin. We hypothesized that CD24 might promote the activation of the Wnt pathway resulting in translocation of β-catenin into the nucleus. We confirmed this by performing nuclear/cytoplasmic fractionation assay where we found higher levels of β-catenin in the nucleus of SW480 cells which were stably transfected with CD24 compared to control cells. More importantly, β-catenin driven luciferase assay has shown that CD24 amplifies the transcriptional activity of β-catenin indicating that it interacts in some way with the wnt signaling pathway to release β-catenin from the destruction complex located in the cytoplasm so it can enter the nucleus. These findings raised the question about how CD24 activates the Wnt signaling. The immunoprecipitation studies have demonstrated that CD24 might interact directly with β-catenin; however, further investigation is needed to determine the precise nature of this interaction and if it is direct or indirect, as well as to identify other putative proteins that might participate in this interplay.

$Apc^{Min/+}/CD24^{-/-}$ (double KO) mice were previously generated in our lab and a significant reduction in the number of polyps was seen [34]. More evidence of the prevention of neoplasia formation in the double KO mice was seen when we checked the hemoglobin and RBC levels in all of the mice. Depletion of even one allele of CD24 in $Apc^{Min/+}$ was sufficient to prevent the anemia and maintain the hemoglobin at a normal level. Accordingly the massive splenomegaly observed in the $Apc^{Min/+}$, due to extra-medullary hematopoiesis, disappeared in the heterozygote state.

In this study, the mice were sacrificed at the age of 24 weeks, and we decided to rely on two different methods for the small and large intestine. For the large intestine a pre-sacrifice colonoscopy was performed, and despite the relatively small number of polyps in the colon of these mice, we saw a statistically significant reduction in the number polyps. For the small intestine, histology was the golden standard. In the small intestine of the $Apc^{Min/+}$ mice, we found about 25 polyps on average. Importantly, depletion of one allele or complete KO of CD24 showed a significant reduction to about 7-8 polyps only. This is more confirmation that CD24 depletion has a preventive effect on neoplasia formation. These finding correspond with *in vitro* results, especially that the $Apc^{Min/+}$ mice bear a mutation in the APC gene that is the typical genetic alteration seen at an early stage of CRC. This means that the Wnt pathway is active in these mice and CD24 might be an excellent target to block this activation as it is also overexpressed at the early stages of the disease.

In conclusion, our findings suggest that; 1. CD24 plays a major role in intestinal tumorigenesis, 2. CD24 interacts with the Wnt pathway by activating β-catenin 3. Knocking out even one copy of CD24 significantly reduces neoplastic formation *in vivo*, and prevents anemia and splenomegaly, the hallmark of intestinal blood loss seeing in the $Apc^{Min/+}$ mice. 4. Downregulation of CD24 may be an important target in the therapy of CRC. Our future work will focus on further investigating the molecular mechanisms underlying these effects and the role of CD24 in intestinal tumorigenesis.

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