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Tumorigenesis and Neoplastic Progression

c-Jun NH_2-Terminal Kinase 1 Plays a Critical Role in Intestinal Homeostasis and Tumor Suppression

Chang Tong,* Zhinan Yin,† Zibo Song,‡ Ashley Dockendorff,‡ Chuanshu Huang,§ John Mariadason,‡ Richard A. Flavell,¶ Roger J. Davis,‖ Leonard H. Augenlicht,‡ and Wancai Yang*

From the Department of Pathology, University of Illinois at Chicago, Chicago, Illinois; the Department of Medicine,† Section of Rheumatology, and the Howard Hughes Medical Institute and Section of Immunobiology,¶ Yale University School of Medicine, New Haven, Connecticut; the Department of Oncology,‡ Montefiore Medical Center, Albert Einstein College of Medicine and Cancer Center, Bronx, New York; the Nelson Institute of Environmental Medicine,§ New York University School of Medicine, Tuxedo, New York; and the Howard Hughes Medical Institute and Program in Molecular Medicine,‖ University of Massachusetts Medical School, Worcester, Massachusetts*

The c-Jun NH_2-terminal kinase (JNK) signal transduction pathway plays important roles in cellular processes and stress. However, the role of JNK1 in intestinal homeostasis and tumorigenesis is unknown. Therefore, we used a JNK1 knockout mouse model to characterize intestinal cell maturation and tumorigenesis. In addition, colon cancer cell lines were used to validate the role of JNK1 and to elucidate the underlying molecular mechanisms *in vitro*. To our surprise, we found that mice with targeted inactivation of JNK1 spontaneously developed intestinal tumors. The normal mucosa in JNK1-deficient mice showed decreased cell differentiation and increased cell proliferation. This tumorigenesis was closely linked to the down-regulation of $p21^{WAF1/cip1}$, a cyclin-dependent kinase inhibitor, in intestinal epithelial cells. Immunohistochemical staining showed that JNK1 was highly expressed in the differentiation compartment of the intestinal mucosa and that the expression of JNK1 was significantly decreased in both human colonic and mouse intestinal tumors. In the colon cancer cell lines, JNK1 expression was up-regulated during spontaneous differentiation, corresponding to the up-regulation of $p21^{WAF1/cip1}$. Moreover, butyrate-induced p21 expression was linked to phosphorylation of JNK1. Reduced JNK1 expression by small interfering RNA suppressed butyrate-induced apoptosis. We concluded that JNK1 plays a critical role in the regulation of homeostasis and in the suppression of tumor formation in the intestine, which was linked to the altered expression of $p21^{WAF1/cip1}$. ***(Am J Pathol 2007, 171:297–303; DOI: 10.2353/ajpath.2007.061036)***

The c-Jun NH_2-terminal kinases (JNKs) are members of the mitogen-activated protein kinase (MAPK) family known to phosphorylate and activate several transcription factors, including c-Jun, ATF-2, activating protein-1 (AP-1), and p53.[1–11] These signal transduction pathways that link to JNK play an important role in a variety of cellular processes, including cell proliferation, differentiation, and apoptosis.[12–14]

The JNKs are encoded by three genes, *JNK1*, *JNK2*, and *JNK3*. JNK1 and JNK2 are ubiquitously expressed, whereas the expression of JNK3 is restricted to the brain and heart.[1,5,15] Increasing evidence shows that JNK1 and JNK2 have distinct functions in the regulation of apoptosis and cell proliferation.[9,14,16–19] Previous reports showed that the targeted inactivation of JNK1 caused decreased T-cell differentiation leading to impaired immunological response[20,21] and that the absence of JNK1 increased skin tumorigenesis in mice initiated by 7,12-dimethylbenz(*a*)anthracene and promoted by 12-*O*-tetradecanoylphorbol-13-acetate.[22] Further, JNK1-deficient mice were highly susceptible to tumor development after inoculation of melanoma cells.[23] In contrast, JNK2 deficiency in mice led to an inhibition of tumorigenesis.[24]

As regards intestinal tumor formation, truncation of c-Jun, which prevents its phosphorylation and activation by JNK, was recently shown to attenuate intestinal tumor formation in $Apc^{Min/+}$ mice.[25] Therefore, we investigated the effect of targeted inactivation of *JNK1*, a ubiquitously

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Address reprint requests to Wancai Yang, M.D., Department of Pathology, University of Illinois at Chicago, 840 S. Wood St., Room 113 CSN, Chicago, IL 60612. E-mail: wyang06@uic.edu.

expressed JNK, in the intestine. We found that the targeted inactivation of *JNK1* spontaneously caused intestinal tumor formation in the mouse, which was linked to alterations in intestinal cell maturation both *in vivo* and in cell culture systems *in vitro*. The alterations in JNK1 expression were closely paralleled in all cases by changes in expression of $p21^{WAF1/cip1}$, a cyclin-dependent kinase inhibitor.

Materials and Methods

$JNK1^{-/-}$ Mice

The generation of the $JNK1^{-/-}$ mouse model and genotyping for *JNK1* has been reported.[21] In brief, an internal 5.5-kb *JNK1* genomic fragment containing four exons was replaced by a PGK-hyg (hygromycin phosphotransferase) cassette. The targeting vector was electroporated into W9.5 ES cells, homologous recombination clones were identified by Southern blot. The homologous recombinant clones were injected into C57BL/6 blastocysts and then were transplanted into pseudopregnant female mice to generate chimeras. The heterozygous (+/−) were intercrossed to generate homozygous, heterozygous, and wild-type mice.[21] The genotyping was identified by polymerase chain reaction. The primers are as follows: for wild-type allele: forward: 5′-GCCATTCTGGTAGAGGAAGTTTCTC-3′, reverse: 5′-CGCCAGTCCAAAATCAAGAATC-3′; for mutant allele: forward: shared with wild-type allele, reverse: 5′-CCAGCTCATTCCTCCACTCATG-3′. The mice that were *JNK1* wild-type, heterozygous, or homozygous were maintained under specific pathogen-free conditions in the animal facility and fed standard chow diet (LabDiet, Somerville, NJ). Mouse dissection, tissue processing, and histopathology were as described previously.[26,27]

Immunohistochemical Staining

Ten pairs of colonic cancers and their adjacent normal mucosa were collected from surgically resected colon cancer patients at Montefiore Medical Center with protocol approval of the institutional review board. Mouse tumors and normal mucosa were from the $Apc^{+/-}$ mice, as reported previously.[28] For immunohistochemical staining of JNK1, 4-μm formalin-fixed and paraffin-embedded sections were deparaffinized and rehydrated, quenched with 1.5% H_2O_2, blocked with 10% normal goat serum, and probed with mouse anti-JNK1 monoclonal antibody (sc-1648; Santa Cruz Biotechnology, Santa Cruz, CA). Detection was with biotinylated anti-mouse IgG, followed by incubation with avidin-biotin complex (Vector Laboratories, Burlingame, CA) and the substrate 3′,5′-diaminobenzidine, combined with hematoxylin counterstaining. Cell proliferation and goblet cell differentiation in intestinal mucosa were assayed by proliferating cell nuclear antigen and Alcian blue staining, respectively, and were counted under microscope, as reported previously.[26]

Cell Lines and Cell Culture

Human colon cancer cell lines Caco-2 and HCT116 were from American Type Culture Collection (Manassas, VA). Human colon cancer cells were maintained in minimal essential medium that was supplemented with 10% fetal bovine serum, 1× antibiotic/antimycotic (100 U/ml streptomycin, 100 U/ml penicillin, and 0.25 μg/ml amphotericin B), 100 μmol/L nonessential amino acids, and 10 mmol/L HEPES buffer solution (all from Invitrogen Corp., Carlsbad, CA). All cells were cultured at 37°C in 5% CO_2. Sodium butyrate (Sigma, St. Louis, MO) was dissolved in 1× phosphate-buffered saline (PBS) for treatment of cell lines.

Western Blot

For the determination of protein expression, cells were seeded in triplicate in six-well plates. Seeding densities varied between 3×10^5 and 6×10^5 cells/well. After overnight culture, cells were treated with 5 mmol/L of sodium butyrate. At 0, 8, 24, and 48 hours after treatment, the cells were harvested and washed twice with ice-cold PBS, and then cell lysis buffer (Cell Signaling Inc., Beverly, MA) was added with incubation on ice for 15 minutes. Cells were harvested by scraping and transferred to a 1.5-ml microcentrifuge tube. After brief sonication, total cell lysate was centrifuged at 10,000 rpm for 10 minutes at 4°C. The supernatant was fractionated by electrophoresis in a 12% sodium dodecyl sulfate-polyacrylamide gel electrophoresis and transferred to a nitrocellulose membrane. Anti-$p21^{WAF1}$, anti-JNK1 (Santa Cruz Biotechnology), anti-phosphorylated-JNK1 (Cell Signal Technologies, Danvers, MA), anti-carcinoembryonic antigen (Sigma), and anti-intestinal trefoil factor (kindly provided by Dr. D.K. Podolsky, Harvard Medical School, Boston, MA)[29–31] antibodies were used as probes. A signal was detected by enhanced chemiluminescence (Amersham Life Science, Piscataway, NJ), as described previously.[27]

Small Interfering RNA Experiments

Targeted small interfering RNA for human JNK1 (si-JNK1; Sigma) (sequence of si-JNK1 was 5′-GGGAUUUGUUAUCCAAAAU-3′) was transfected into the human colon cancer cell line HCT116, followed by treatment with sodium butyrate for 24 hours. The knockout efficiency was detected by reduction of JNK1 protein level. Apoptosis was measured by flow cytometry with propidium iodide staining as we described recently.[32] The alteration of JNK1 and phosphorylated JNK1 were assayed by Western blot.

Isolation of Epithelial Cells from Mouse Intestine and Western Blot

A 50-ml conical tube filled with 25 ml of 15 mmol/L ethylenediaminetetraacetic acid was prewarmed at 37°C in a shaking incubator for at least 15 minutes. The mouse intestine was removed and placed in a Petri dish filled with ice-cold PBS (Mg^{2+}- and Ca^{2+}-free). The intestine was opened longitudinally and cleaned with ice-cold PBS and then cut into small pieces (2 to 3 cm) using a scalpel.

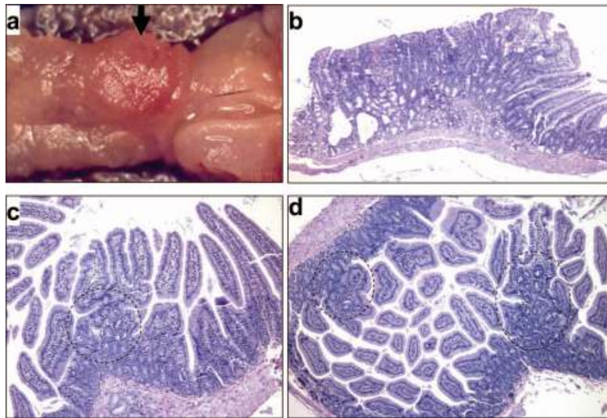

Figure 1. Target inactivation of *JNK1* causes intestinal tumor formation in mouse. **a:** A big tumor (**arrow**) in the duodenum of $JNK1^{-/-}$ mouse (gross pathology); **b:** histopathology confirmed that the tumor in **a** was an adenoma; **c:** another duodenal polyp was found in the same mouse as in **a** by histopathology; and **d:** two polyps seen in the duodenum of another $JNK1^{-/-}$ mouse.

The pieces of intestine were then transferred to the prewarmed 50-ml conical tube and incubated in the conical tube for 20 minutes at 37°C with continuous shaking. The tube was vortexed for 30 seconds or until the tissue appeared mostly clear and solution was cloudy with cells. The remaining tissues (submucosa and muscle) were removed with forceps. The cell solution was spun in a refrigerated centrifuge at 3500 rpm for 5 minutes to get a cell pellet and then resuspended with ice-cold PBS and spun an additional 5 minutes. The PBS was discarded as much as possible; the appropriate amount of lysis buffer was then added to a tube to extract protein for Western blot. Anti-p21 antibody was used to determine alteration of p21 in intestinal epithelial cells, anti-intestinal trefoil factor polyclonal antibody was used to determine the alteration of cell differentiation, anti-JNK1 antibody was used for the confirmation of genotype, and anti-β-action antibody was used as internal control.

Coimmunoprecipitation

Coimmunoprecipitation was performed by using the Catch and Release System (Upstate Biotechnology, Lake Placid, NY) as instructed by the manufacturer. In brief, 500 μg of total cell lysate, 2 μg of anti-p21 antibody, and 10 μl of antibody-affinity matrix were mixed and transferred to the Catch and Release column. For control, 2 μg of anti-Muc1 antibody was added instead of anti-p21 antibody. The mixture was incubated for 3 hours at 4°C with continuous agitation. After washing five times with 1× washing buffer, the immunocomplexes were eluted by adding 70 μl of elution buffer. The eluted sample was fractionated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis, and Western blot analysis was used to detect JNK1 with anti-JNK1 antibody (Santa Cruz Biotechnology). As a control, p21 antibody was probed to the blot.

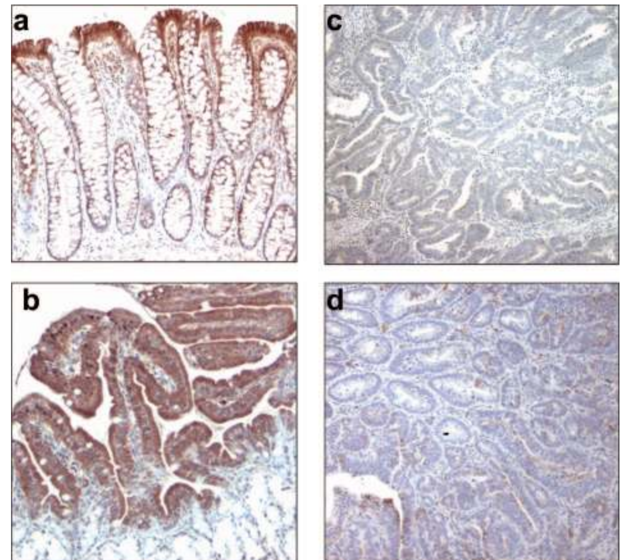

Figure 2. JNK1 is highly expressed in the differentiation compartment of normal mucosa, but its expression is decreased in human and mouse tumors, by immunohistochemical staining with anti-JNK1 antibody. **a:** Human normal colon mucosa; **b:** mouse normal small intestine; **c:** paired human colon cancer; and **d:** mouse intestinal tumor.

Results

Mice with targeted deletion of *JNK1* spontaneously developed small bowel tumors. Figure 1a shows a large tumor in the duodenum of a 4-month $JNK1^{-/-}$ mouse, which was characterized histologically as an adenoma (Figure 1b). A second duodenal adenoma was found in the same mouse (Figure 1c). Figure 1d shows that a 3-month-old mouse developed two duodenal adenomas. In total, 64% of 17 $JNK1^{-/-}$ mice sacrificed at age 3 to 11 months spontaneously developed tumors in the small intestine, at a frequency of 0.86 ± 0.21 tumors per mouse (mean ± SD). All of these tumors were adenomas, no invasive adenocarcinoma was observed. In contrast, there was no intestinal tumor formation in the mice that were wild-type (10 mice) or heterozygous (10 mice) for *JNK1.*

The surprising observation that deletion of *JNK1* could lead to intestinal tumor formation prompted study of the role of this gene in the normal intestine. Human and mouse tissues were evaluated immunohistochemically for JNK1 expression. JNK1 was highly expressed in the upper portion of human colonic crypts (Figure 2a) and in the villi of the mouse duodenal mucosa (Figure 2b), localized in the differentiation apartment. However, there was a striking loss of JNK1 expression in a corresponding human colonic adenocarcinoma (Figure 2c) and in a duodenal adenoma from an $Apc^{+/-}$ mouse (Figure 2d).[26] Thus, loss of JNK1 expression occurs in human and mouse intestinal tumors (ie, Figure 1).

The normal expression of JNK1 in the villi and upper crypts of the small and large intestine, respectively, suggested that JNK1 was linked to maturation of intestinal epithelial cells. This was supported by observations that, in the duodenal mucosa of $JNK1^{-/-}$ mice compared with wild-type mice, the number of goblet cells, visualized by Alcian blue staining, was signifi-

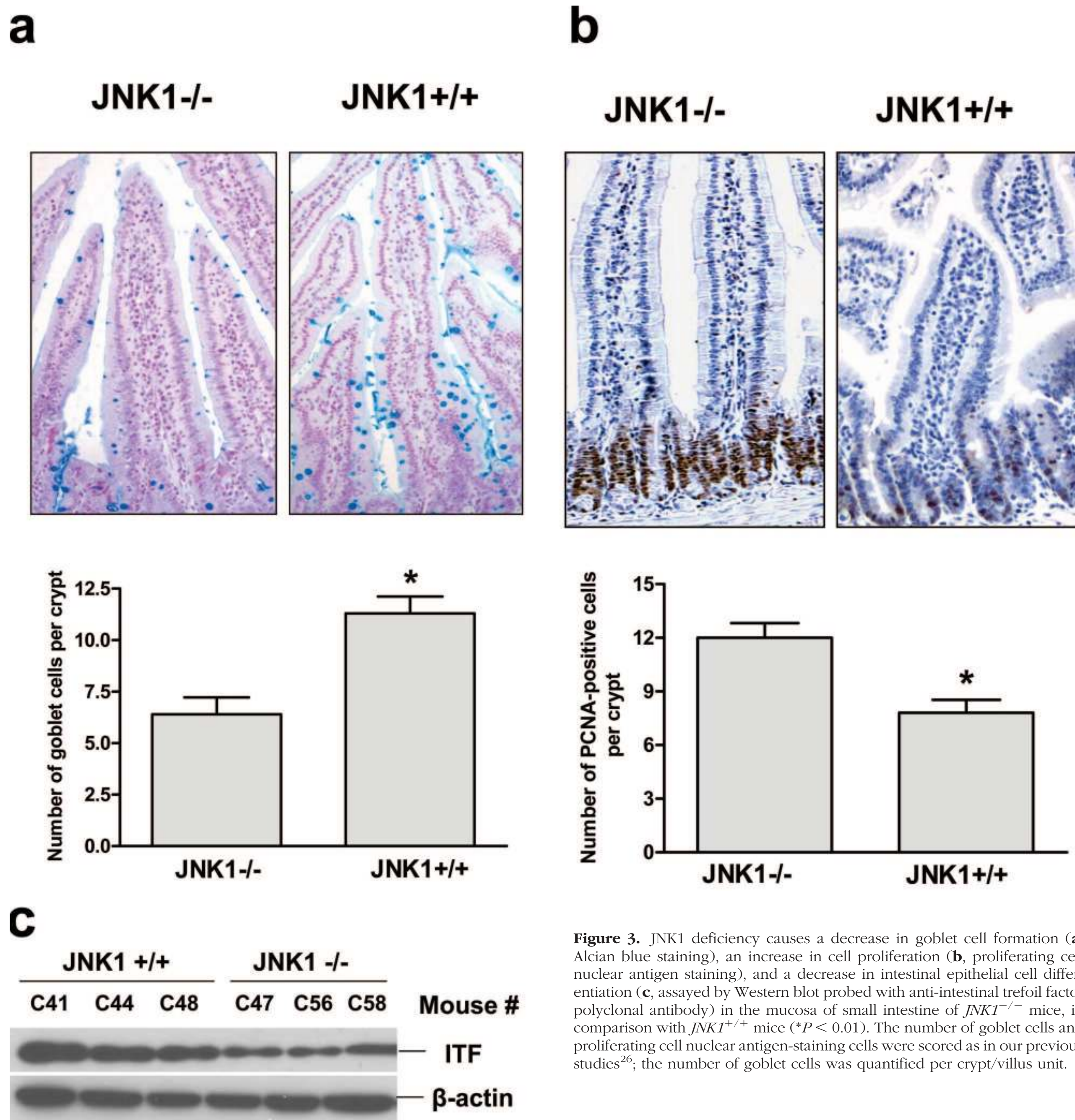

Figure 3. JNK1 deficiency causes a decrease in goblet cell formation (**a**, Alcian blue staining), an increase in cell proliferation (**b**, proliferating cell nuclear antigen staining), and a decrease in intestinal epithelial cell differentiation (**c**, assayed by Western blot probed with anti-intestinal trefoil factor polyclonal antibody) in the mucosa of small intestine of *JNK1*$^{-/-}$ mice, in comparison with *JNK1*$^{+/+}$ mice (*$P < 0.01$). The number of goblet cells and proliferating cell nuclear antigen-staining cells were scored as in our previous studies[26]; the number of goblet cells was quantified per crypt/villus unit.

cantly decreased ($P < 0.01$) (Figure 3a). This was consistent with the down-regulation of intestinal trefoil factor expression in the epithelial cells of *JNK1*$^{-/-}$ mice in comparison with *JNK1*$^{+/+}$ mice (Figure 3c). However, the number of proliferating cells detected by proliferating cell nuclear antigen staining was significantly increased (Figure 3b).

To dissect this link of JNK1 to intestinal cell maturation, we turned to cell culture model systems. The human colonic cancer cell lines Caco2 spontaneously undergoes cell cycle arrest and differentiation.[33,34] During the time course of these maturation programs, the expression of JNK1 was increased (Figure 4). Moreover, this was coincident with increased p21$^{WAF1/cip1}$ and carcinoembryonic antigen expression. This parallels the coexpression of JNK1 and p21$^{WAF1/cip1}$ in the differentiated compartment of the small and large intestine *in vivo* (Figure 2).[26,27,35]

To determine whether these increases in JNK1 and p21$^{WAF1/cip1}$ were functionally linked, we induced maturation of the colonic epithelial cell line HCT116 with the short-chain fatty acid butyrate, a physiological inducer of colonic cell maturation.[36] As shown in Figure 5a, sodium butyrate induced a significant increase in p21 expression in HCT116 cells by 8, 24, and 48 hours, corresponding to the increased phosphorylation of JNK1 (p-JNK1, Figure 5a), there was no increase in total JNK1 expression (Fig-

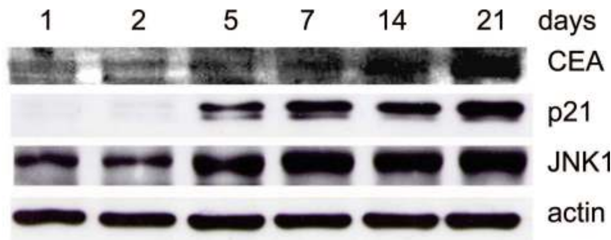

Figure 4. JNK1 expression is increased during human colon cancer cell spontaneous differentiation (in terms of increase in carcinoembryonic antigen expression). The increase in JNK1 was correlated with the up-regulation of $p21^{WAF1/cip1}$ in human colon cancer cell lines Caco2. β-Actin was used as loading control.

ure 5a). Because p21 could be regulated by multiple factors that were stimulated by butyrate, induction of p21 was seen earlier, after 8 hours of treatment, than phosphorylated-JNK1 (Figure 5a). However, in $JNK1^{-/-}$ mice, in which intestinal cell maturation was impaired (Figure 3), and in which tumors form (Figure 1), there was a significant decrease in p21 expression in the intestinal epithelial cells (Figure 5b).

To determine the requirement for JNK1 in butyrate-induced apoptosis in human colon cancer cells, targeted small interfering RNA for human JNK1 (si-JNK1) was transfected into the human cancer cell line HCT116 to reduce JNK1 expression, followed by treatment with sodium butyrate for 24 hours. As shown in Figure 5c, butyrate significantly induced apoptosis in HCT116 cells after 24 hours of treatment. However, the induction of apoptosis was suppressed by 40% in the cells transfected with si-JNK1, compared with the cells transfected with control small interfering RNA (si-control, sequence was 5′-AUGAACGUGAAUUGCUC AA-3′). The knockdown efficiency was confirmed by Western blot (Figure 5d). Western blot also showed that JNK1 and phosphorylated-JNK1 (p-JNK1) were significantly induced by sodium butyrate; however, once JNK1 was knocked down, p-JNK1 was down-regulated, and induction of both JNK1 and p-JNK1 by butyrate was much lower than that in the HCT116 cells without si-JNK1 transfection or with si-control transfection (Figure 5d), corresponding to the suppression of apoptosis.

Our most recent work, as a complementary study, has shown that JNK1 expression was down-regulated in HCT116 cells that were *p21*-null ($p21^{-/-}$ HCT116 cells) in comparison with *p21*-wild-type HCT116 cells ($p21^{+/+}$ HCT116).[32] Because the expression of JNK1 and $p21^{WAF1/cip1}$ were closely associated and were functionally linked in the mouse intestine and in butyrate-induced apoptosis in culture, we asked whether there was a physical interaction between these molecules that may underlie a functional interaction. An endogenous immunocomplex formed by incubation of a p21 antibody and a lysate from HCT116 cells was positive for JNK1 (Figure 6, lane 5). The similar interaction of JNK1 and p21 was seen in colon cancer cell line Caco2 (data not shown), which was consistent with the previous report.[37] Thus, not only are JNK1 and $p21^{WAF1/cip1}$ closely linked in expression both *in vitro* and *in vivo*, but they physically interact as well.

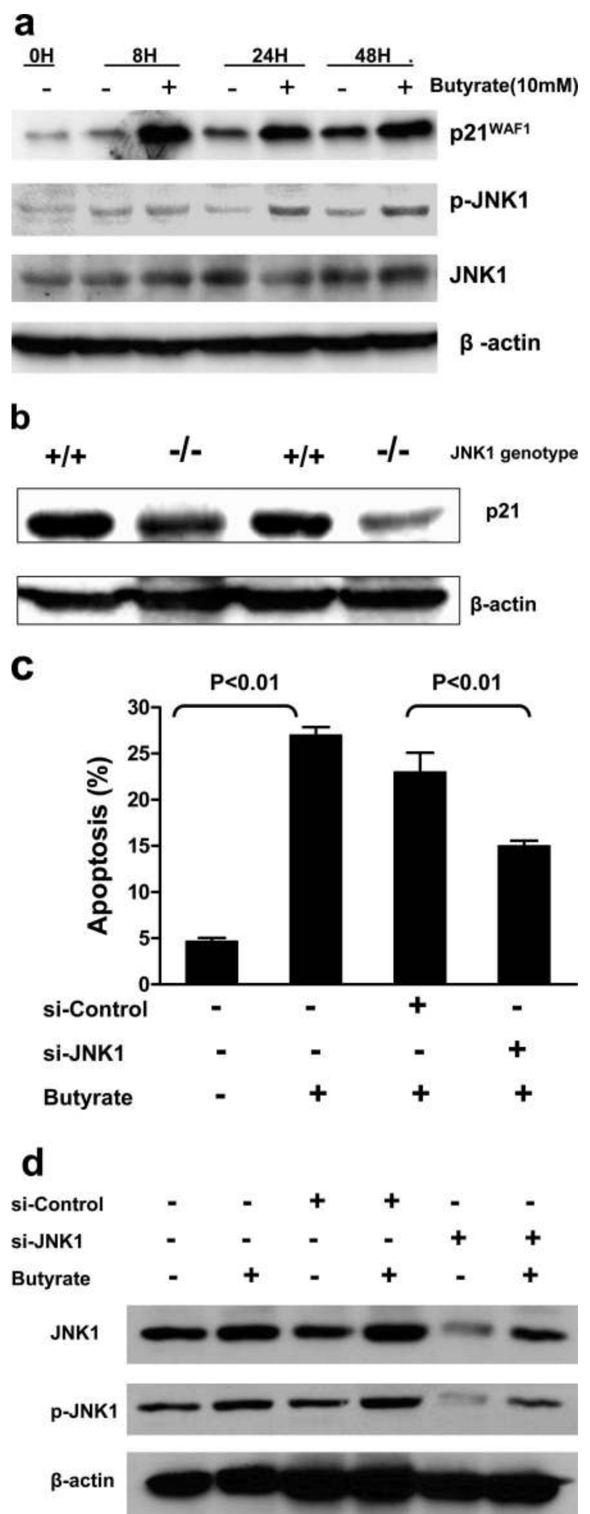

Figure 5. Butyrate-induced apoptosis is JNK1-dependent in human colon cancer cell line HCT116, which is associated with the induction of p21. **a:** Sodium butyrate-induced p21 expression, also induced phosphorylation of JNK1 (p-JNK1); **b:** p21 was down-regulated in the intestinal epithelial cells of $JNK1^{-/-}$ mice, compared wtih $JNK^{+/+}$ mice (assayed by Western blot); **c:** targeted small interfering RNA for human JNK1 (si-JNK1) significantly suppressed butyrate-induced apoptosis, compared with the cells transfected with control small interfering RNA (si-Control); and **d:** both JNK1 and phosphorylated-JNK1 (p-JNK1) were induced by butyrate, but once JNK1 was knocked down, p-JNK1 was down-regulated too, and the induction of JNK1 and p-JNK1 by butyrate was much less than that in HCT116 cells with si-control transfection. β-Actin was used as internal control.

IP: Anti-p21

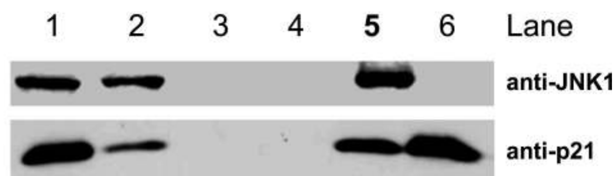

Figure 6. Coimmunoprecipitation: JNK1 physically binds to p21$^{WAF1/cip1}$. HCT116 cell lysate was subjected to immunoprecipitation with anti-p21 antibody, and the immunocomplex was probed for the presence of JNK1 with anti-JNK1 antibody. **Lane 1**, total cell lysate, used as positive control; **lane 2**, column flow through; **lane 3**, washed one time; **lane 4**, washed five times; **lane 5**, immunocomplex; and **lane 6**, negative control.

Discussion

Our data demonstrate that JNK1 plays a critical role in regulating homeostasis and in suppressing tumorigenesis in the intestine. Loss of JNK1 causes defects in regulation of intestinal proliferation and lineage-specific differentiation that leads to development of intestinal tumors, which was linked to the altered expression of p21, *in vivo* and *in vitro*.

The cdk inhibitor p21 plays an important role in intestinal homeostasis and tumor formation: loss of p21 was detected early in colon tumor formation,[35] the absence of p21 has been linked to the inability of colon cancer cells to arrest in the G_1 phase of cell cycle,[38] the cell cycle arrest and apoptosis of colon cancer cells stimulated by the short-chain fatty acid butyrate and the nonsteroidal anti-inflammatory drug sulindac are linked to the induction of p21,[39–42] and inactivation of *p21* can increase and accelerate tumor formation initiated by *Apc* or *Muc2* gene deficiency.[26,27] Further, we report here that in the intestinal epithelial cells of *JNK1*$^{-/-}$ mice, p21 was dramatically down-regulated (Figure 5b). However, because inactivation of p21 is not able to initiate tumor formation,[38] tumors that arise in *JNK-1*-deficient mice are not solely attributable to alterations in p21 expression and/or function. Nevertheless, our data on the temporal, functional and physical interaction between p21$^{WAF1/cip1}$ and JNK1 suggest that at least some of their functions in regulating intestinal epithelial cell maturation are through a complex that includes both molecules.

Like *ApcMin*$^{+/-}$,[44] *Apc1638*$^{+/-}$[28] and other animal models (ie, Apc/p21 models[26]), *JNK1* knockout mouse model developed tumors mostly localized in the small bowel, not in the large bowel, which are different from human intestinal cancers that mostly developed in the colon and rectum. We postulated that genetic background and environmental factors (diets) play important roles in initiating and localizing tumor formation in entire gastrointestinal tract. For example, all *ApcMin*$^{+/-}$, *Apc1638*$^{+/-}$, and *JNK1*$^{-/-}$ models are in Black6 background[21,28,43] and were usually fed rodent chow diet, the majority of tumors were formed in small intestine, but once Black6 mice carried a mutant allele of Pla2g2s,[44] the mice were more susceptible to both small intestine and colon tumors. When Apc models (including *Apc/p21* and *Apc/p27* compound models[26,45]) were fed high-fat, low-calcium, and low-vitamin D diet that mimics major risk factors for colorectal cancer in the United States and other Western countries, tumors were seen in both the small and large bowels. One of the things to consider is that people with familial adenomatous polyposis develop small intestine tumors, generally in the duodenum, and they are a common occurrence now that more people are surviving colorectal cancer. There are some hereditary nonpolyposis colorectal cancer families, and some members of these families had tumors in the stomach and small intestine, some members had tumors in large intestine, and some members had tumors in entire gastrointestine, even though the patients had similar mismatch repair genes mutation (eg, hMLH1 and hMSH2).[46,47] This also suggests that the environment plays an important role because that is one feature that changed throughout several decades. It has been described previously that *JNK1* deficiency caused an adverse effect on immune function via impairing T-cell differentiation.[21] Whether impaired immune function caused by loss of JNK1 contributed to intestinal tumor formation in this mouse model is not clear and is under investigation.

Whether the perturbation of intestinal homeostasis and intestinal tumor formation in *JNK1*$^{-/-}$ mice can be explained by known functions of JNK1 and p21, or interactions among the known complexity of signaling pathways that regulate intestinal cell maturation is not clear. However, the data demonstrate the key role of JNK1 in regulation of the maturation of intestinal epithelial cells and in suppression of intestinal tumor formation. Moreover, the expression of JNK1 and p21 was linked in tissue culture systems of intestinal cell maturation, both molecules are overexpressed in the differentiated compartment of the intestinal crypt, and they formed a physical complex. Whether there are regulatory interactions or whether both JNK1 and p21 are regulated by a common pathway or pathways remains to be determined.

Acknowledgments

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