Cyclin D1 Is Not an Immediate Target of β-Catenin following Apc Loss in the Intestine*

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Cyclin D1 is postulated to be a target of the canonical Wnt pathway and critical for intestinal adenoma development. We show here that, unlike cyclin D1 reporter assays, endogenous cyclin D1 levels are not affected following antagonist of the Wnt pathway in vitro, nor is cyclin D1 immediately up-regulated following conditional loss of Apc in vivo. Cyclin D1 levels do, however, increase in a delayed manner in a small subset of cells, suggesting such up-regulation occurs as a secondary event. We also analyzed the immediate consequences of Apc loss in a cyclin D1−/− background and failed to find any cyclin D1-dependent phenotypes. However, we did observe elevated cyclin D1 expression in lesions developing 20 days after Apc loss. In these circumstances, all adenomas (but not smaller lesions) showed cyclin D1 up-regulation. Finally in a smaller study, we analyzed whether cyclin D1 deficiency affected adenoma formation 20 days following induced loss of Apc. Unlike AhCre+ Apcfl/fl mice (which all developed adenomas), doubly mutant AhCre+ Apcfl/fl cyclin D1−/− mice only developed small lesions. Taken together, this argues that cyclin D1 up-regulation in intestinal neoplasia is important for tumor progression rather than initiation.

The APC1 gene encodes the adenomatous polyposis coli tumor suppressor protein, germ line mutation of which characterizes familial adenomatous polyposis, an autosomal syndrome characterized by multiple colorectal lesions (1). Inactivation of APC is also recognized as a key early event in the development of sporadic colorectal cancers (2). It is becoming increasingly clear that the mechanism by which Apc mediates tumor suppression is primarily by targeting β-catenin for degradation and thereby regulating Wnt signaling (3). The immediate cellular consequences of Wnt deregulation include perturbed proliferation, migration, and differentiation in the murine small intestine (4).

Cyclin D1 has been proposed as a Wnt target gene because β-catenin has been shown to regulate cyclin D1 in colorectal cancer cell lines in vitro (5, 6). Consistent with this, cyclin D1 overexpression has been observed in human colorectal cancers and in adenomas arising in the ApcMin/+ mouse (7, 8). However, several recent transcriptome studies have failed to confirm cyclin D1 as an immediate target of perturbed Wnt signaling (4, 9). Furthermore, cyclin D1 is not essential for the development of intestinal tumorigenesis because ApcMin/+ cyclin D1−/− mice still develop adenomas, albeit at a lower frequency (10, 11). Indeed, in some settings, loss of cyclin D1 can actually accelerate tumorigenesis (for example, following activation of β-catenin signaling within the mammary gland) (12). These studies have cast doubt on the timing and significance of the up-regulation of cyclin D1 following loss of Apc. We have therefore investigated the up-regulation cyclin D1 in vivo following conditional loss of Apc and activation of β-catenin. Furthermore we have investigated the significance of loss of cyclin D1 to the early stages of intestinal tumorigenesis.

Experimental Procedures

Luciferase Reporter Assay—To measure TCF/β-catenin-driven transactivation, a luciferase reporter assay was performed using TCF reporter constructs TOPFLASH and FOPFLASH (9). TOPFLASH contains TCF binding sites upstream of a herpes simplex virus thymidine kinase promoter, driving luciferase. The control plasmid FOPFLASH carries mutated TCF binding sites. The cyclin D1 promoter constructs have been described previously and were a kind gift of Dr. F. McCormick (5). For transient transfections, 250,000 cells/well were seeded in 6-well plates and transfected with 500 ng of the indicated luciferase reporter and 50 ng of TKRenilla (Promega) using FuGENE 6 (Roche Applied Science). Expression of dnTCF was induced by the addition of doxycycline to a final concentration of 1 μg/ml (9). Luciferase activity was determined using the dual luciferase reporter assay system (Promega) 24 h after induction.

Cell Lines—All cells were grown in RPMI 1640 medium supplemented with 10% fetal calf serum and antibiotics. Colo 205 and LS174T cells carrying inducible dnTCF have been described previously (9). In short, L5174T cells carrying mutated β-catenin and DLD1 cells carrying mutated APC were transfected with pCDNA6TR (Invitrogen). Stable clones with high levels of tetracycline repressor activity were subsequently transfected with pCDNA4TO-dnTCF1 or pCDNA4TO-dnTCF4 to generate inducible dnTCF cell lines.

Maintenance and Induction of Cre Recombinase in Mice—All experiments were performed according to UK Home Office regulations. Mice were segregating for the C57BL6/J, 129/Ola, and C3H genomes at a ratio of 75%, 12.5%, and 12.5%, respectively. Siblings were used as controls. AhCre− mice were intercrossed with mice carrying a LoxP-flanked Apc allele (Apcfl/fl), Rosa 26 LacZ reporter allele (4, 13), and a cyclin D1−/− allele (14). For induction of Cre recombinase, mice were given three intraperitoneal injections of 80 mg/kg β-napthoflavone and infected with AhCre+ mice 3, 4, and 5 days later. To achieve the later time points of 6 and 8 days, mice received a single intraperitoneal injection of 80 mg/kg β-napthoflavone, which yielded levels of recombination of ∼30% (15). To achieve adenoma formation, mice were fed a dietary supplement of 0.8% β-napthoflavone for 3 days. This was then removed, and mice

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‡ The abbreviations used are: APC, adenomatous polyposis coli; TCF, T cell factor; dnTCF, dominant negative TCF.
were left for 20 days or longer until they developed adenomas. LacZ wholemount staining was performed as described in Ireland et al. (15).

Immunohistochemistry—Cyclin D1 immunohistochemistry was performed using mouse monoclonal antibody DCS-6 at a dilution of 1:100 (Nova Castra), and antigen retrieval was performed using a high pH target retrieval solution from Dako at 99 °C for 30 min. β-Catenin immunohistochemistry was performed as described in Sansom et al. (4). Alcian blue staining was performed as described in Sansom et al. (4).

Northern Blotting—Total RNA (10 μg) was separated on 1.25% agarose and transferred to Zeta-Probe Membrane (Bio-Rad). Loading was checked by EtBr staining. Hybridization was performed using ExpressHyb Hybridization Solution (Clontech). Indicated probes were labeled using RadPrime DNA labeling system (Invitrogen).

Assaying Apoptosis and Proliferation—Apoptosis was scored from hematoxylin and eosin-stained sections as previously described in Sansom et al. (4). Proliferation was scored through S phase labeling; mice received injection 2 h before harvesting with 0.25 ml of bromodeoxyuridine (Amersham Biosciences), and bromodeoxyuridine immunohistochemistry was performed (4).

RESULTS

Inhibition of Wnt Signaling Does Not Cause Reduction in Endogenous Levels of Cyclin D1 in Vitro—Previous studies by Tetsu and McCormick (5) and Shtutman et al. (6) have proposed cyclin D1 as a direct Wnt target gene within colorectal cancer cell lines. However, both of these studies used promoter reporter constructs to measure increases in cyclin D1 rather than examining endogenous cyclin D1 expression. To directly test whether results from these promoter reporter constructs reflect regulation of the gene by β-catenin, we studied endogenous cyclin D1 levels and two cyclin D1 luciferase reporters following inhibition of β-catenin signaling. This was achieved using colorectal cancer cell lines LS174T and DLD1, which express an active mutant β-catenin protein and a truncated APC protein, respectively, and carry doxycycline-inducible expression plasmids encoding N-terminally truncated versions of TCFs. These dnTCF proteins act as potent inhibitors of the endogenous β-catenin/TCF-driven transactivation present in colorectal cancer (9). We have previously shown that following induction of dnTCF4 or dnTCF1, there is an inhibition of β-catenin signaling that induces a rapid cell cycle arrest and down-regulation of a number of Wnt target genes including MYC, BMP4, and EPHB3. However, no changes in levels of cyclin D1 RNA were detectable in either cell line 24 h following induction of either dnTCF1 or dnTCF4 (Fig. 1A). Using the same two cyclin D1 promoter reporter constructs (−1748CD1) and (−962CD1) used by Tetsu and McCormick (5), luciferase activity was measured following dnTCF4 induction in LS174T cells. Unlike endogenous levels of cyclin D1, which stay constant, an inhibition of cyclin D1 promoter activity as measured by both reporter constructs was observed following dnTCF4 induction (Fig. 1B).

Cyclin D1 Is Not Immediately Up-regulated following Apc Loss in Vivo—Given the failure to see up-regulation of endogenous cyclin D1 in vitro, we next investigated the consequences of Wnt deregulation upon cyclin D1 levels in vivo. To achieve this, a Cre-Lox-based murine system was used to delete Apc in the adult murine intestine. In this system, deletion of Apc is mediated by exposure to β-naphthoflavone, which induces expression of Cre recombinase through activation of a CYP1A1 promoter (4). In conjunction with an Apc allele carrying LoxP sites flanking exon 14 (Apc exon 14 (ApcΔE14)18), this allows inducible inactivation of Apc. This system delivers near 100% recombination of the target allele in adult murine small intestine (4).

We have previously performed a microarray to analyze the expression profiles of adult small intestine tissues 4 and 5 days following the loss of Apc (4). This analysis failed to reveal an up-regulation of cyclin D1 at either time point. Unlike β-catenin, which was nuclear in every cell (Fig. 2, A and B, inset), immunohistochemical analysis of cyclin D1 at 4, 6, and 8 days following Apc loss in AhCre- Apcfl/fl mice (Fig. 2, A–D) showed up-regulation in only a small number of cells at the leading edge of the phenotypic abnormalities, where Apc-deficient cells are directly juxtaposed to Apc-positive cells on the villus (see Sansom et al. (4) for Apc staining).

Cyclin D1 Deficiency Does Not Alter the Immediate Phenotypes of Apc Deficiency in Vivo—The failure to see up-regulation of cyclin D1 raised the possibility that early phenotypes associated with Apc deficiency are cyclin D1-independent. Previously, we have shown that conditional deletion of Apc in the murine small intestine leads to marked increases in crypt size, proliferation (as determined through bromodeoxyuridine incorporation), and apoptosis, together with an abrogation of migration and differentiation (4).

We therefore intercrossed cyclin D1-deficient mice with AhCre- Apcfl/fl mice to investigate whether cyclin D1 deficiency modified any of these immediate phenotypes. Fig. 3 shows that cyclin D1 deficiency did not rescue the increase in crypt size, the enhanced and position-independent proliferation, or the induction of apoptosis seen following Apc loss. These features are therefore all independent of cyclin D1. Hulit et al. (11) have recently reported that ApcMin/+ mice show a relative deficiency of goblet cells and that cyclin D1 deficiency restores the goblet cell number to wild type levels. We have previously shown that in the absence of Apc, there is a complete block on goblet cell differentiation (4). Therefore, we assessed whether this phenotype could be rescued by cyclin D1 deficiency. However, no Alcian blue staining was observed in the AhCre- Apcfl/fl cyclin D1+/− crypts, showing that rescue was not occurring. This failure to restore goblet cell differentiation presumably reflects a dose dependence, with hemizygosity for Apc in the previous study leading to perturbed rather than blocked differentiation, a state that can be rescued by cyclin D1 deficiency.
FIG. 2. Cyclin D1 up-regulation in a subset of cells in induced AhCre+ Apcfl/fl mice. A, no cyclin D1 staining in control AhCre− Apcfl/fl intestine at day 4 post-induction. The arrow indicates false positive staining of endothelial cells. B, cyclin D1 staining in induced AhCre+ Apcfl/fl mice at day 4. Insets for A and B show β-catenin staining; note nuclear β-catenin staining in all cells in the AhCre+ Apcfl/fl crypts. The arrow denotes a small area of cells with nuclear cyclin D1 staining. C and D, induced AhCre+ Apcfl/fl mice at day 6 and 8, respectively, show many more nuclei with cyclin D1 up-regulation, concentrated toward the leading edge of aberrance.

FIG. 3. Deficiency of cyclin D1 does not alter the immediate phenotypes of Apc deficiency. A–D, hematoxylin and eosin staining showing enlarged crypts in both AhCre+ Apcfl/fl cyclin D1+/+ (C) and AhCre+ Apcfl/fl cyclin D1−/− crypts (D) compared with wild type (A) and cyclin D1−/− crypts (B). E–H, bromodeoxyuridine immunohistochemistry showing increased S-phase labeling in the crypts of AhCre+ Apcfl/fl cyclin D1+/+ (G) and AhCre+ Apcfl/fl cyclin D1−/− (H) compared with wild type (E) and cyclin D1−/− crypts (F). I–L, Alcian blue staining for goblet cells showing reduced numbers in both AhCre+ Apcfl/fl cyclin D1+/+ (K) and AhCre+ Apcfl/fl cyclin D1−/− crypts (L) compared with wild type (I) and cyclin D1−/− crypts (J). M, apoptosis is up-regulated in both AhCre+ Apcfl/fl cyclin D1+/+ (gray bars) and AhCre+ Apcfl/fl cyclin D1−/− crypts (black bars) compared with wild type (blue bars) and cyclin D1−/− crypts (white bars). No significant difference was observed between Cre+ Apcfl/fl cyclin D1+/+ and Cre+ Apcfl/fl cyclin D1−/− crypts (p = 0.8, Mann-Whitney, n = 4).
Cyclin D1 Is Not a Direct Wnt Target Gene

Cyclin D1 Is Up-regulated in All Adenomas and Required for Efficient Adenoma Formation—Because studies have shown that cyclin D1 is up-regulated in adenomas arising in the ApcMin/+ mouse, we next investigated the profile of cyclin D1 up-regulation in a range of lesions. To achieve this, we modified our Cre induction procedure so that mice were fed with a dietary supplement of 0.8 mg/ml β-napthoflavone for 3 days. This causes a lower level of Cre-mediated recombination (Fig. 4, A–K). Within the smaller number of recombined crypts, this protocol leads to an identical phenotype at day 4 to that observed following high efficiency recombination (i.e. perturbed differentiation and migration as described above). Importantly, this lower recombination protocol allowed the development of a range of intestinal lesions and adenomas 20 days after induction in AhCre+/Apcfl/fl mice (Fig. 4, G–F). This spectrum of lesions observed at this time point was similar to those we have classified previously from the ApcMin/+ mouse (16), ranging from a single dysplastic crypt to adenomas.

This range of lesion types developing within an individual mouse allowed us to profile the pattern of cyclin D1 up-regulation. Fig. 4, G–J, shows the pattern of cyclin D1 expression in the different lesion types observed in AhCre+/Apcfl/fl 20 days after induction of Cre recombinase. Small lesions (n = 30; Fig. 4, G and H) showed a similar pattern of cyclin D1 up-regulation to the Apc-deleted intestine at days 4–8, with a small number of cells up-regulating cyclin D1. All adenomas (n = 10) showed up-regulation of cyclin D1 in virtually all the epithelial cells (Fig. 4J).

Finally, to test whether this up-regulation of cyclin D1 was important for efficient tumorigenesis, we investigated adenoma formation in AhCre+/Apcfl/fl cyclin D1+/− mice 20 days after induction. All AhCre+/Apcfl/fl cyclin D1+/− mice developed a minimum of 20 adenomas within this time period (n = 8; Fig. 4C); however, none of the AhCre+/Apcfl/fl cyclin D1+/− mice developed adenomas over the 20-day period. Instead, AhCre+/Apcfl/fl cyclin D1+/− mice developed much smaller lesions (no greater than 1 mm in diameter) over this time period that were only able to be visualized on wholemount sections due to the presence of the Rosa 26 R LacZ reporter (Fig. 4K).

DISCUSSION

Cyclin D1 Is Not an Immediate Wnt Target Gene in Vitro or in Vivo—Our failure to see changes in endogenous cyclin D1 levels both in vitro and in vivo indicates that cyclin D1 is not an immediate target gene of β-catenin. This contrasts with data obtained using cyclin D1 reporter constructs in the same cells in vitro, indicating that strategies reliant solely on reporter construct assays do not always accurately reflect changes in endogenous gene expression levels and so may lead to an over-estimation of the number of direct Wnt target genes.

The definitive evidence that cyclin D1 is not an immediate target gene came from the in vivo data, in which cyclin D1 was up-regulated in a delayed manner in a small subset of Apc-deficient cells that juxtaposed wild type cells. Notably, up-regulation of cyclin D1 has previously been reported within the leading edge of human colorectal adenocarcinomas (17). These findings suggest that the additional factors required to up-regulate cyclin D1 may derive from the interaction between wild type and Apc-deficient cells. This contrasts with a number of immediate Wnt target genes (such as C-MYC and CD44) that we have previously shown to be up-regulated coincidentally with nuclear relocalization of β-catenin (4).

A number of genes such as brachyury have been shown to require TCF/lymphoid enhancer factor signaling for maintai-
nance rather than initiation of expression (18). Cyclin D1 may fit into this class of genes that has TCF/lymphoid enhancer factor binding sites but requires other factors to initiate expression because dysregulation of β-catenin expression per se is not enough to immediately up-regulate cyclin D1.

Cyclin D1 Is Important for Efficient Adenoma Formation—Unlike the very early stages of intestinal tumorigenesis, we found that cyclin D1 was up-regulated in all the adenomas of AhCre+ Apcfl/fl mice. This consolidates previous data showing up-regulation of cyclin D1 in adenomas from the ApcMin/+ mouse and also implicates cyclin D1 in tumor growth rather than initiation. Our failure to find any adenomas in the Apcfl/fl cyclin D1−/− mice at 20 days is also strongly consistent with the notion that cyclin D1 is required for efficient intestinal tumorigenesis. However, cyclin D1 deficiency slows rather than stops this process, and it seems likely that the numerous small lesions in the double-null mice may progress to adenomas, albeit in a delayed time frame. This interpretation is consistent with the documented reduction of adenoma formation in ApcMin/+ cyclin D1−/− mice (10, 11) and also with the delayed and lesion-specific pattern of cyclin D1 up-regulation reported here.

Single knockouts of cyclin D1, D2, and D3 show very restricted abnormalities and are characterized by up-regulation of the other family members (19). In contrast, when mice are generated with only one functional cyclin D family member, this leads to early mortality due to localized abnormalities in tissues unable to up-regulate the remaining cyclin D family member. Indeed, our array analysis has indicated that cyclin D1 is immediately up-regulated following Apc loss (22). Given this possible up-regulation of cyclin D2, our data therefore suggest that therapies targeted against the cyclin D proteins should not be restricted to cyclin D1 alone.

Taken together, our data indicate that cyclin D1 is not a direct target of Wnt signaling and that cyclin D1 is not important for the very early stages of intestinal tumorigenesis. Indeed, a recent study has also shown that cyclin D1 is not up-regulated in all intestinal cells following Apc loss (22). Consistent with this, deficiency of cyclin D1 does not modify any of the immediate phenotypes associated with Apc loss. However, it is clear that cyclin D1 does become rapidly deregulated as a secondary event and that cyclin D1 status is an important factor in adenoma establishment and growth.

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