Cancer Genes and Genomics

Molecular Cancer Research

The MIF Homologue D-Dopachrome Tautomerase Promotes COX-2 Expression through β-Catenin–Dependent and –Independent Mechanisms

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Abstract

The cytokine/growth factor, macrophage migration inhibitory factor (MIF), contributes to pathologies associated with immune, inflammatory, and neoplastic disease processes. Several studies have shown an important contributing role for MIF-dependent COX-2 expression in the progression of these disorders. We now report that the MIF homologue, D-dopachrome tautomerase (D-DT), is both sufficient and necessary for maximal COX-2 expression in colorectal adenocarcinoma cell lines. D-DT–dependent COX-2 transcription is mediated in part by β-catenin protein stabilization and subsequent transcription. Also contributing to D-DTs regulation of COX-2 expression are the activities of both c-jun-N-terminal kinase and the MIF-interacting protein, Jab1/CSN5. Interestingly, D-DT–dependent β-catenin stabilization is regulated by COX-2 expression, suggesting the existence of an amplification loop between COX-2- and β-catenin–mediated transcription in these cells. Because both COX-2- and β-catenin–mediated transcription are important contributors to colorectal cancer (CRC) disease maintenance and progression, these findings suggest a unique and novel regulatory role for MIF family members in CRC pathogenesis. *Mol Cancer Res; 8(12); 1601–9. ©2010 AACR.*

Introduction

The development of colorectal cancer (CRC) arises from the sequential accumulation of mutations or deletions in the coding sequence of a number of tumor suppressor genes and oncogenes (1). One of the most commonly mutated tumor suppressors in CRC is the adenomatous polyposis coli (APC) gene, which normally controls the levels and activity of Wnt-dependent transcription through β-catenin phosphorylation/degradation, of protumorigenic gene products (1, 2). One such gene product of β-catenin/TCF (T-cell factor/lymphoid enhancer factor)-dependent transcription is COX-2, a well-described target of the Wnt pathway that is generally accepted as playing a contributory role in colorectal adenocarcinoma initiation and progression (3–5). COX-2 is 1 of 2 isoforms of prostaglandin H2 synthase and acts to catalyze the synthesis of eicosanoids and prostaglandins from arachidonic acid. It promotes CRC tumorigenesis and neoplastic maintenance through effects of its metabolites on angiogenesis, apoptosis, and tumor cell invasiveness (6–8). Accordingly, selective COX-2 inhibitors suppress the growth of tumor cells *in vitro* and tumor growth and maintenance *in vivo* (9, 10). However, clinical studies in humans reveal that COX-2 antagonists also induce phenotypic changes in human vascular smooth muscle cells that increase the risk of myocardial infarction and other thrombotic cardiovascular events (11).

The proinflammatory and mitogenic cytokine, macrophage migration inhibitory factor (MIF), has been found to be an important endogenous mediator of COX-2 expression in a number of different cell types and is necessary for several proinflammatory and protumorigenic activities of the MIF (12–15). Unlike other cytokines, MIF also has the unique ability to catalyze a *nonphysiologic* enzymatic reaction (16). It converts D-dopachrome—a stereoisomer of dopachrome not present in mammals—into 5,6-dihydroxyindole-2-carboxylic acid. The only known MIF homologue, D-dopachrome tautomerase (D-DT), not only retains this tautomerase activity but also decarboxylates the D-dopachrome substrate to give a 5,6-dihydroxyindole product (17). Although D-DT retains only 38% identity and 49% homology to MIF, the tertiary structure of D-DT is remarkably similar (18). Despite these intriguing similarities to the well-studied MIF, there are virtually no reports on the biologic function(s) of D-DT. Early studies describing the enzymatic activity and molecular cloning reveal that D-DT is relatively highly expressed in heart, brain, spleen, lung, skeletal muscle, kidney, and testes whereas liver expression seems to be the highest (17). Although no prior studies

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investigating D-DT report on its expression in the colon, findings described herein indicate that D-DT is highly expressed in 2 human CRC cell lines.

We recently showed that D-DT cooperates with MIF in dictating the steady-state expression of the proangiogenic growth factors, VEGF and IL-8, in non–small cell lung cancer (NSCLC) cell lines (19). Angiogenic growth factor expression mediated by endogenous D-DT relies upon a c-jun-N-terminal kinase (JNK)/activator protein 1 (AP-1)-dependent signaling pathway. In the present study, we investigated the contribution and mechanism of D-DT to COX-2 expression in colorectal adenocarcinoma cells. We report herein that both β-catenin–dependent and β-catenin–independent pathways are utilized by D-DT in regulating COX-2 expression in human colorectal adenocarcinoma cells.

Materials and Methods

Cells and reagents

HCT-116, HT-29, and HeLa cell lines were purchased from ATCC and grown in Dulbecco's modified Eagle medium supplemented with 10% heat-inactivated FBS, L-glutamate, and gentamycin. Myc-tagged wild-type and mutant human β-catenin mammalian expression constructs were kindly provided by Dr. Frank McCormick (University of California San Francisco) (20). Dr. Curtis C. Harris (National Cancer Institute, NIH), kindly provided us with wild-type, mutant, and deletion human COX-2 promoter luciferase constructs. TOPFlash and FOPFlash reporter plasmids were from Promega. Antibodies used for immunoblotting include polyclonal and monoclonal antibodies directed against MIF (Santa Cruz Biotechnology and R&D Systems, respectively), D-DT (19), V5 (Sigma), β-catenin (BD Transduction Laboratories), COX-2 (Cayman Chemical), β-actin (Sigma), CSN5 (Bethyl Laboratories), and α-tubulin (Sigma).

RNA interference

shRNA design software from Dharmacon si*DESIGN* Center (www.dharmacon.com/sidesign/) was used to design shRNA sequences. MIF, D-DT, and CSN5 oligos (MIF: 5′-CCTTCTGGTGGGGAGAAAT-3′; D-DT#2: 5′-GCCAGGACCGGATACTTAT-3′; Jab1/CSN5: 5′-GCTCAGAGTATCGATGAAA-3′) were ordered from Dharmacon (Thermo Scientific). Commercially available shRNA directed against β-catenin was from Santa Cruz Biotechnology. shRNA oligos were transfected into cells by using Oligofectamine following manufacturer's directions (Invitrogen; refs. 19, 21). As negative controls, both a commercially available control shRNA (Dharmacon) and a scrambled shRNA based on the sequence of D-DT shRNA were used accordingly (both referred to as nonspecific, Scr).

Quantitative PCR

For total RNA isolation, the RNeasy Mini Kit (Qiagen) was used following the manufacturer's protocol, followed by cDNA synthesis using Omniscript RT (Qiagen). Quantitative PCR was carried out on a DNA Engine Opticon Thermocycler (BioRad), using Takara PCR mix (Takara Bio Inc.), 0.3 μmol/L of forward and reverse primers (Invitrogen), and SYBr Green (Molecular Probes) diluted to a ratio of 1:25,000. Primers used were as follows:

COX-2: Forward 5′-CTAGAGCCCTTCCTCCTGTG-3′
Reverse 5′-GGGGATCAGGGATGAACTTT-3′
D-DT: Forward 5′-AGAACCGCTCCTACAGCAAG-3′
Reverse 5′-TAGGCGAAGGTGGAGTTGTT-3′
β-Actin: Forward 5′-CAAGGCCAACCGCGAGAAGA-3′
Reverse 5′-GGATAGCACAGCCTGGATAG-3′

Relative expression levels of mRNAs were determined using the ΔCt method. The ΔΔCt was calculated as the difference between the normalized Ct values (ΔCt) of the treatment and the control samples: $\Delta\Delta Ct = \Delta Ct_{treatment} - \Delta Ct_{control}$. ΔΔCt was then converted to fold change by the following formula: fold change $= 2^{-\Delta\Delta Ct}$.

Western blotting

For whole-cell extracts, cells were lysed in 1× RIPA buffer, harvested by scraping, and homogenized with a 23-gauge needle on a 1-mL syringe. Protein concentrations were determined by the DC Protein Assay kit (BioRad). Equal concentrations of protein from indicated lysates were run on precast 10% or 4% to 20% SDS-polyacrylamide gels (BioRad), followed by transfer to polyvinylidene difluoride membranes (Millipore). Membranes were blocked in blocking buffer (5% nonfat dried milk, 0.2% Tween-20, 1% goat serum in TBS), incubated with primary antibody in the same solution, washed 3 × 5 minutes in wash buffer (TBS + Tween-20), reincubated with secondary antibody at a 1:8,000 dilution in blocking buffer, washed again, and visualized with ECL reagent (Pierce). TIFF images of scanned blots were analyzed by Scion Image (Scion Corp.).

Luciferase assay

Cells (4×10^4/mL) were plated in a 24-well plate and transfected with shRNA the following day for an additional 48 hours. Cells were then transiently cotransfected with 0.125 μg/well of TOPFlash or FOPFlash luciferase promoter plasmids together with 0.0125 μg/well *Renilla* pRL-null plasmid (Promega), using Lipofectamine (Invitrogen) transfection reagent. After 24 hours, *Firefly* and *Renilla* luciferase activities were measured by the Dual Luciferase Reporter Assay System (Promega) on a TD-20/20 luminometer (Turner Designs).

Adenovirus

Adenovirus for D-DT was prepared as described previously (19). β-Catenin and constitutively active MKK7 recombinant adenoviruses were purchased from Vector Biolabs and were used to infect cells at approximately 5×10^7 virus particles/mL. COX-2 adenovirus was generated using the Gateway cloning system (Invitrogen). Briefly, human COX-2 was PCR amplified and TOPO cloned into the pENTR D-TOPO plasmid. LR Recombinase

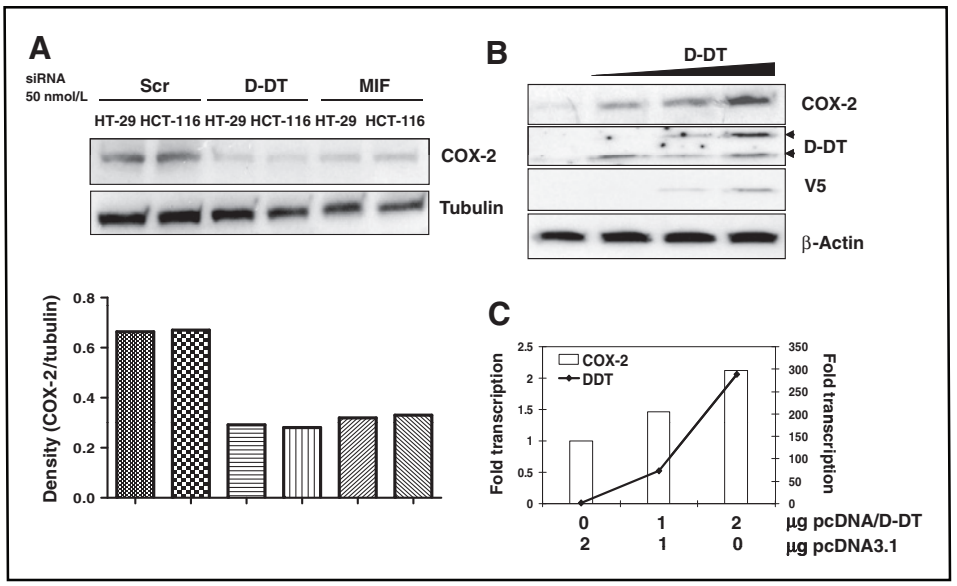

Figure 1. Regulation of COX-2 expression by D-DT. A, human CRC cell lines, HCT-116 and HT-29, were plated in 6-well plates for 24 hours, followed by oligotransfection of 50 nmol/L of scrambled, nonspecific shRNA oligos, 50 nmol/L of D-DT–specific shRNA oligos, or 50 nmol/L of MIF-specific shRNA oligos as described (19, 21). After 48 hours, lysates were prepared and equal amounts of protein were analyzed by immunoblotting for COX-2 and α-tubulin (loading control). Scion Image was used for densitometry and COX-2/tubulin densitometry values are shown. Data are representative of 4 independent experiments. B, HT-29 cells were transfected with a D-DT overexpression construct in a dose-dependent manner (lane 1, 4 μg of pcDNA3.1 vector alone; lane 2, 1 μg of pcDNA3.1/D-DT; lane 3, 2 μg of pcDNA3.1/D-DT; lane 4, 4 μg of pcDNA3.1/D-DT). Thirty-six hours later, equal concentrations of protein lysates were analyzed by immunoblotting for COX-2, D-DT, V5-epitope tag, and β-actin. Data are representative of 2 independent experiments. C, different concentrations of pcDNA3.1/D-DT expression construct (0, 1, or 2 μg of pcDNA3.1/D-DT and 2, 1, or 0 μg of pcDNA3.1 vector alone to control for plasmid concentration) were transfected into HT-29 cells. Forty-eight hours later, total RNA was isolated and reverse transcribed to cDNA. Real-time PCR was used to determine the relative quantities of mRNA in the indicated samples. Data shown represent the ΔCt of the average of duplicate reactions for each condition between target mRNA (D-DT and COX-2) and β-actin and are representative of 2 independent experiments.

(Invitrogen) was used to shuttle inserts into the pAd/CMV/V5-DEST destination vector, and subclones were confirmed by sequencing. Adenoviral vectors were digested with PacI, ethanol precipitated, and transfected into 293A adenoviral packaging cells by using Lipofectamine (Invitrogen). After amplifying viral supernatants, virus was purified using Virabind purification columns (Cell Biolabs) and tested for expression efficiency versus toxicity.

Statistical analyses

Results were expressed as mean ± SEM. Data comparisons were derived by unpaired, 2-tailed *t* tests, using GraphPad Prism 4.1 statistical program. The values of $P < 0.05$ were considered significant.

Results

COX-2 regulation by D-DT

Because the macrophage MIF homologue D-DT cooperates with MIF in regulating the steady-state expression of angiogenic growth factors in lung adenocarcinoma cells, we investigated whether D-DT similarly regulates COX-2 expression, as has been reported for MIF (12–14). Specifically, we examined the potential role for D-DT–dependent expression of COX-2 in human CRC cell lines. HT-29, a grade II human colon adenocarcinoma cell line (mutant APC), and HCT-116, a human colorectal carcinoma cell line (ref. 22; wild-type APC), were transfected with scrambled, D-DT, or MIF shRNA oligos as described in the Materials and Methods section. As shown in Figure 1A, shRNA-mediated knockdown of either MIF or D-DT in HT-29 and HCT-116 cells results in significant reductions in COX-2 expression accompanying approximately 70% to 80% reductions in MIF and D-DT protein levels (19) and *data not shown*.

To investigate whether D-DT is *sufficient* to promote COX-2 expression, HT-29 cells were transfected with varying concentrations of a D-DT mammalian expression construct or vector control alone, and COX-2 protein levels were evaluated. As shown in Figure 1B, COX-2 protein levels increased proportionally to increasing D-DT in D-DT–transfected cells versus control plasmid–transfected cells alone. Furthermore, D-DT overexpression resulted in increased transcription from a COX-2 promoter–luciferase reporter plasmid (Fig. 1C). Combined, these results suggest that COX-2 is a transcriptional target for D-DT.

D-DT promotes β-catenin stabilization and transcriptional activity

Because β-catenin–mediated transcription contributes to COX-2 expression (3, 4), we next tested whether D-DT regulates β-catenin levels and/or its transcriptional activity. As shown in Figure 2A, whole-cell lysates from cells rendered deficient in D-DT by shRNA show reduced protein levels of both COX-2 and β-catenin. Moreover, D-DT–deficient cells had approximately 50% less

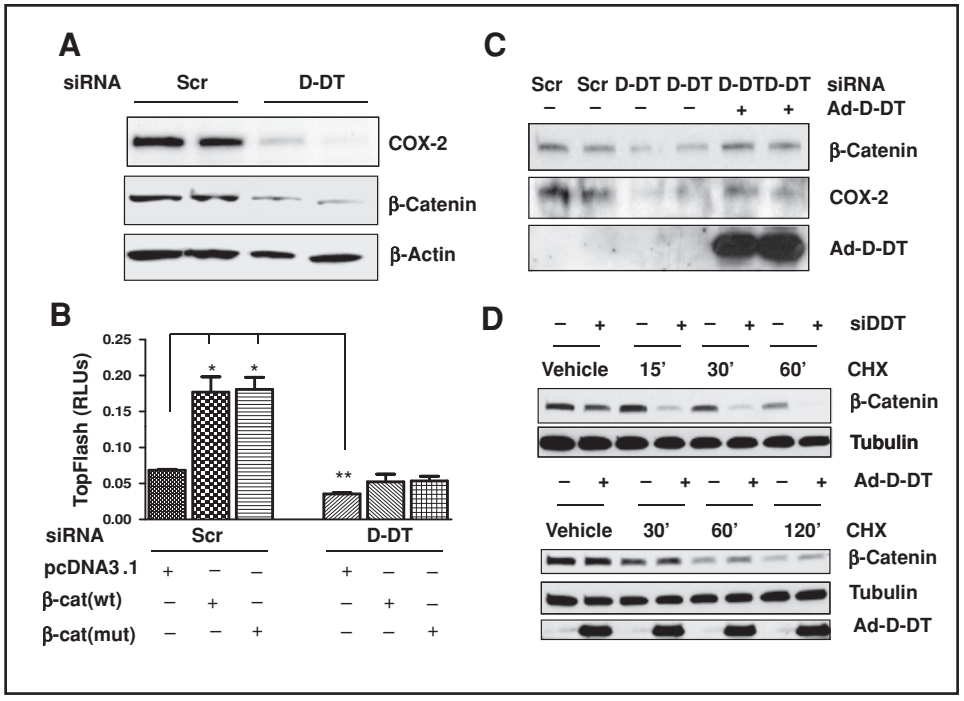

Figure 2. Reduced β-catenin expression and activity in D-DT–deficient cells. A, HT-29 cells were plated in 6-well plates for 24 hours, followed by oligotransfection with 50 nmol/L of scrambled shRNA oligos or 50 nmol/L of D-DT–specific shRNA oligos for an additional 48 hours. Lysates were prepared and equal amounts of protein were analyzed by immunoblotting for COX-2, β-catenin, and β-actin (loading control). B, 24 hours after transfection with Scr or D-DT shRNA, cells were cotransfected with the β-catenin/TCF reporter plasmids pTOP*Flash*/pRL-null (*Renilla*) alone or together with vector control (pcDNA3.1), wild-type [pcDNA3.1/β-catenin, β-Cat(wt)], or phosphorylation of mutant β-catenin [pcDNA3.1/β-catenin^{mut}, β-Cat(mut)] expression constructs. Twenty-four hours later, *Firefly* and *Renilla* luciferase activities were measured by the Dual Luciferase Reporter Assay System. Results are expressed as relative light units (RLUs) after normalizing ratios of luciferase/*Renilla* luciferase from triplicate samples. Parallel transfections with the β-catenin/TCF nonresponsive reporter control construct pFOP*Flash* revealed equally, negligible RLUs in all cells regardless of D-DT or β-catenin status (*not shown*). C, cells were treated as in A and 24 hours after shRNA transfection, D-DT adenovirus [(+)Ad-D-DT] was used to infect D-DT shRNA transfected cells and GFP adenovirus was used for the remainder of the cells [(−)Ad-D-DT] for an additional 48 hours. Lysates were prepared and equal amounts of protein were analyzed by Western blot for COX-2, β-catenin, and D-DT. D, cells were treated with 20 μg/mL of cycloheximide for the indicated times 48 hours after transfection with Scr or D-DT shRNA (top) or 24 hours after infection with Ad-GFP [(−)Ad-D-DT] or Ad-D-DT [(+)Ad-D-DT]. At the indicated times following treatment with cycloheximide, cells were lysed and equal amounts of protein from each sample were analyzed by immunoblotting. All results are representative of at least 3 independent experiments.

endogenous β-catenin/TCF-dependent transcription as assessed by a β-catenin/TCF reporter plasmid (Fig. 2B, first bars in series). Importantly, ectopic overexpression of β-catenin in control shRNA-transfected cells resulted in a approximately 2.5-fold increase in ectopically expressed β-catenin/TCF reporter plasmid activity whereas D-DT–deficient cells had only a nominal increase in β-catenin–dependent transcription (Fig. 2B, middle bars in series). Interestingly, overexpression of a phosphorylation defective β-catenin construct resulted in a similar increase in ectopic β-catenin–dependent transcription in D-DT–containing cells as expected, but D-DT–deficient cells were largely resistant, suggesting that the defect in ectopically expressed β-catenin–mediated transcription observed in D-DT knockdown cells is independent of APC/GSK3β-mediated phosphorylation (Fig. 2B, last bars in series).

To rule out the possibility that the reduced COX-2 and β-catenin protein levels in D-DT–deficient cells were independent of the loss of D-DT, we reintroduced D-DT by adenoviral infection. Also note that adenoviral-mediated expression of D-DT, as opposed to adding recombinant D-DT extracellularly, was used throughout these studies to recapitulate the D-DT expression in CRC cells with respect to both intra- and extracellular compartments. Because the D-DT homologue MIF has functional activities both inside and outside of cells (23), we sought to ensure that if D-DT has similar functional properties it would be able to carry out both by expressing it as it would be found normally in cells. As shown in Figure 2C, both COX-2 and β-catenin protein levels were largely restored to control shRNA–transfected cell levels in D-DT knockdown cells infected with adenoviral D-DT. These results validate D-DT as being responsible for the effects of D-DT shRNA on COX-2 and β-catenin expression.

The ability of β-catenin to contribute to nuclear TCF-dependent transcription depends, in large part, on the relative ability of E3-ubiquitin ligase complexes to recognize and ubiquitylate cytoplasmic β-catenin, thus leading to its degradation (24). To investigate whether decreased β-catenin levels and transcriptional activity observed in D-DT–deficient cells were due to decreased β-catenin protein stability, we determined the relative degradation of β-catenin

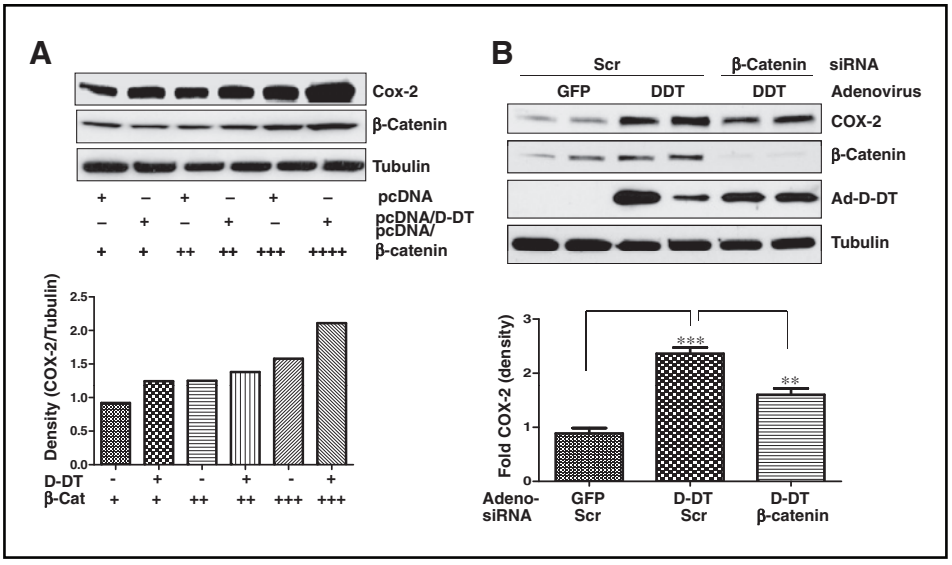

Figure 3. D-DT–mediated β-catenin stabilization contributes to, but is not wholly responsible for, D-DT–dependent COX-2 expression. A, cells were plated in 6-well plates and were transfected the following day with pcDNA3.1/D-DT or pcDNA3.1/β-catenin expression constructs, alone or in combination. For all plasmids, one "+" correlates to 1 μg of construct transfected into cells. The total amount of DNA transfected into cells was equalized by cotransfecting vector control, pcDNA3.1. Forty-eight hours posttransfection, cell lysates were assessed by immunoblotting for COX-2 and β-catenin. Densitometry of COX-2 and tubulin were determined and graphed as COX-2/tubulin. B, cells were transfected with Scr or β-catenin shRNA oligos and were infected with Ad-GFP or Ad-D-DT 24 hours later as indicated. Forty-eight hours postinfection, cells were lysed and assessed by immunoblotting for COX-2, β-catenin, D-DT, and α-tubulin (loading control). Densitometry of COX-2 Western blots from 2 independent experiments was determined and fold COX-2/tubulin expression is shown.

in D-DT–overexpressing and -deficient cells. Following the addition of the protein translation inhibitor cycloheximide to scrambled or D-DT shRNA–transfected HT-29 cells, total β-catenin protein levels were assessed by immunoblotting. As shown in Figure 2D (top), β-catenin expression goes down dramatically faster in D-DT–deficient cells than in control cells. Accordingly, cells infected with D-DT adenovirus displayed slightly higher β-catenin stability than GFP (green fluorescent protein)-infected cells (Fig. 2D, bottom), suggesting that endogenous D-DT levels are, in fact, important for maintaining β-catenin stability. It should be noted that the ability of ectopically expressed D-DT to stabilize β-catenin and induce COX-2 expression is generally modest, suggesting that endogenous D-DT levels are near their contribution threshold.

We next determined whether D-DT–dependent modulation of β-catenin stability contributes to COX-2 expression in CRC cell lines. Because both endogenous and ectopically expressed D-DTs (albeit modestly) stabilize β-catenin protein levels, we overexpressed increasing amounts of β-catenin in the presence or absence of ectopically expressed D-DT. As shown in Figure 3A, HT-29 cells were transfected with empty vector or D-DT along with increasing amounts of β-catenin, and COX-2 expression was evaluated. Although β-catenin transfection, in the absence of ectopically expressed D-DT, was sufficient for enhancing COX-2 expression over basal levels, the combination of D-DT with β-catenin significantly increased COX-2 levels above that seen with ectopically expressed β-catenin alone. This result supports our hypothesis that D-DT acts to stabilize β-catenin, thereby increasing its pool of transcriptionally active nuclear β-catenin.

To further investigate whether D-DT–mediated regulation of enhanced β-catenin expression contributes to D-DT–induced COX-2, D-DT was overexpressed in the presence or absence of β-catenin and COX-2 induction was determined. As shown in Figure 3B, adenoviral-delivered D-DT increased both COX-2 and β-catenin levels in control shRNA–transfected cells as expected. Interestingly, shRNA knockdown of β-catenin in D-DT–overexpressing cells resulted in a reduction of D-DT–induced COX-2 expression by only approximately 50%. This finding indicated to us that β-catenin might not be the only point of control in D-DT–dependent COX-2 expression in CRC cells.

Regulation of JNK/c-jun pathway activation by D-DT

Our laboratory recently reported that D-DT is an integral regulator of the JNK signaling pathway leading to c-jun phosphorylation and angiogenic growth factor expression in lung adenocarcinoma cells (19). Because c-jun/AP-1 transcription is a well-described pathway leading to COX-2 transcription (25–27), we next sought to investigate whether D-DT–dependent c-jun activation contributes to, along with β-catenin, D-DT–induced COX-2 expression. From Figure 4A and consistent with our earlier findings (19), D-DT–deficient cells have reduced levels of phosphorylated c-jun (Fig. 4A) whereas cells infected with Ad-D-DT results in a substantial increase in c-jun

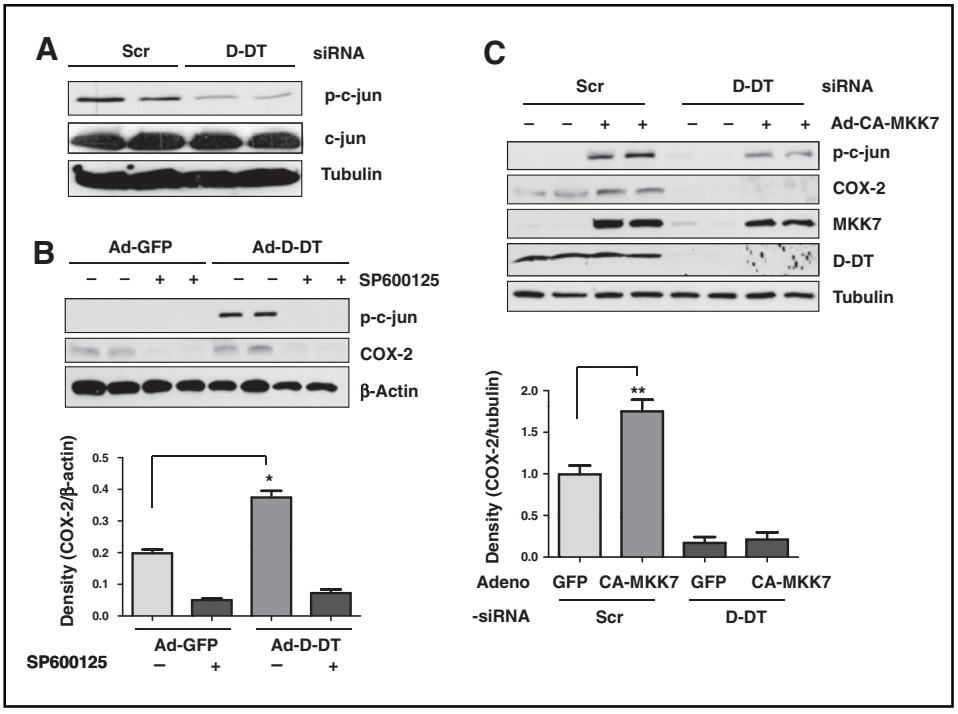

Figure 4. JNK/c-jun is necessary for COX-2 expression in CRC cells and D-DT contributes to JNK/c-jun activation pathway. A, cells were oligotransfected with Scr and D-DT shRNA oligos for 48 hours, lysed, and immunoblotted for phospho-c-jun, total c-jun, and α-tubulin. B, cells were infected with Ad-GFP or Ad-D-DT adenovirus for 24 hours and then treated with 40 μmol/L of SP600125 for an additional 6 hours. Cells were harvested, lysed, and immunoblotted for phospho-c-jun, COX-2, and β-actin. Densitometry of COX-2/β-actin is shown. C, HT-29 cells were transfected with Scr or D-DT shRNA, and 24 hours later cells were infected with Ad-GFP or constitutively active MKK7 adenovirus (Ad-CA-MKK7) as indicated. After 48 hours, cells were harvested, lysed, and immunoblotted for phospho-c-jun, COX-2, MKK7, D-DT, and α-tubulin. Densitometries of COX-2 and tubulin were determined from 2 independent experiments and graphed as COX-2/tubulin.

phosphorylation compared with cells infected with Ad-GFP (Fig. 4B). Importantly, cells treated with the JNK small molecule inhibitor SP600125 abolished both steady-state (Ad-GFP–infected) and Ad-D-DT–induced COX-2 expression (Fig. 4B). Combined, these findings strongly suggest that phosphorylated c-jun is a central participant in CRC COX-2 expression.

To further investigate the contribution of c-jun activation in D-DT–mediated COX-2 expression, we overexpressed a constitutively active mutant of MKK7 (CA MKK7), the upstream kinase of JNK, in D-DT–deficient cells. As shown in Figure 4D, overexpression of CA MKK7 results in increases in both phosphorylated c-jun and COX-2 expression in control shRNA–transfected cells. Interestingly, CA MKK7 increased c-jun phosphorylation levels, although at slightly lower levels than that observed in control cells, but had no effect on rescuing defective COX-2 expression in D-DT–deficient cells. These results suggested to us that phosphorylated c-jun–mediated transcription, likely by the AP-1 transcription factor complex, was somehow defective in D-DT–deficient cells.

Kleeman and colleague showed that the D-DT homologue MIF regulates AP-1–mediated transcriptional activity by functionally regulating the MIF-binding protein, Jab1 (Jun activation domain binding protein-1)/CSN5 (28). In an effort to determine whether Jab1/CSN5 might be implicated in the apparent requirement for D-DT in CA MKK-7–induced COX-2 expression, even though c-jun is adequately phosphorylated, we investigated the relative requirements for Jab1/CSN5 in steady-state CRC COX-2 expression. As shown in Figure 5A, shRNA knockdown of CSN5 results in a dramatic reduction of COX-2 expression in CRC cells mirroring the effects of JNK inhibition (Fig. 5B). We next sought to determine whether there was a requirement for Jab1/CSN5 in D-DT–induced COX-2 expression, as would be predicted if Jab1/CSN5 is, in fact, necessary for AP-1 transcriptional activation of COX-2. As shown in Figure 5B, D-DT stimulates COX-2 expression in control shRNA–transfected cells but CSN5–deficient cells are entirely refractory to D-DT–induced COX-2 expression despite a robust stimulation of c-jun phosphorylation (Fig. 5B, top). Similarly, but not unexpectedly, CA MKK7 could not promote COX-2 expression in Jab1/CSN5-deficient cells (Fig. 5C). Combined, these results suggest that there is a functional requirement for Jab1/CSN5 in D-DT–induced AP-1 activity on COX-2 transcriptional expression. Although beyond the scope of these studies, experiments are currently underway to evaluate the binding and transactivation properties of AP-1 on the human COX-2 promoter in the context of D-DT and/or Jab1/CSN5 manipulation.

Finally, in an effort to evaluate how D-DT influences β-catenin stability, we reexpressed COX-2 by using an adenoviral

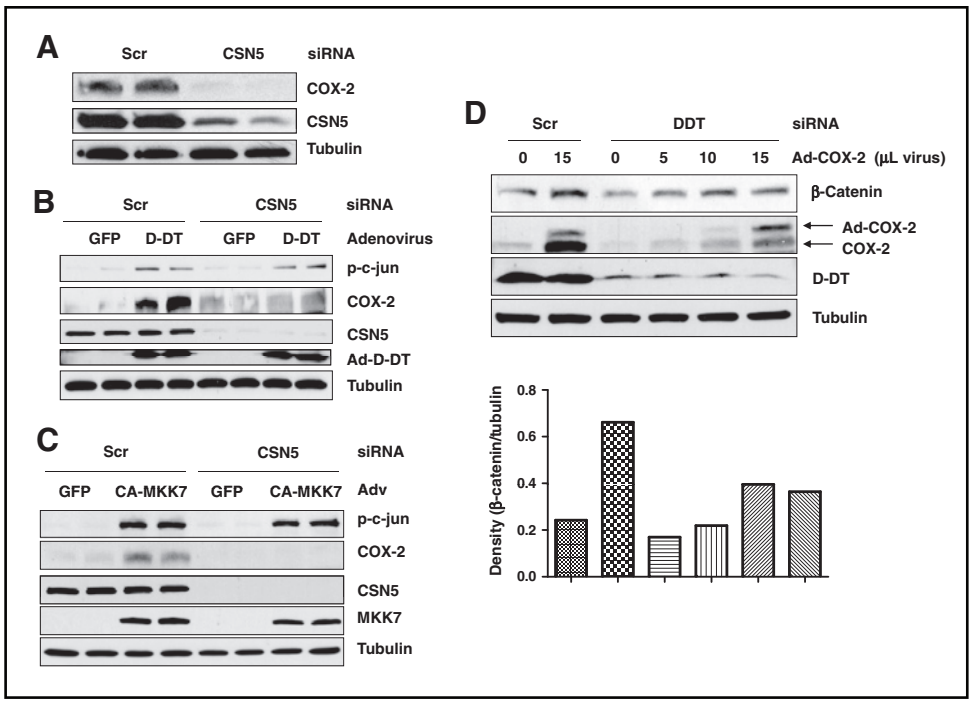

Figure 5. CSN5 participates in the regulation of COX-2 and β-catenin. A, nonspecific control (Scr) and CSN5-specific shRNA–transfected cells were harvested, lysed, and immunoblotted for COX-2, CSN5, and α-tubulin. B, 24 hours after transfection with Scr or CSN5 shRNA oligos, cells were infected by either Ad-GFP or Ad-D-DT for an additional 48 hours. Cells were then harvested, lysed, and immunoblotted for phospho-JNK, phospho-c-jun, COX-2, CSN5, D-DT, and α-tubulin. C, cells were treated as in B but were infected with Ad-GFP and Ad-CA-MKK7 adenoviruses. Forty-eight hours after infection, cells were harvested, lysed, and immunoblotted for phospho-c-jun, COX-2, CSN5, MKK7, and α-tubulin. D, cells were oligotransfected with Scr or D-DT shRNA oligos, and 24 hours later, cells were infected with Ad-GFP or Ad-COX-2 adenovirus as indicated (for 0 μL of Ad-COX-2, cells were treated with Ad-GFP at 15 μL and Ad-GFP was coinfected with Ad-COX-2 at 5 and 10 μL to give 15 μL of total adenovirus for each sample infected). Forty-eight hours after infection, cells were lysed and immunoblotted for β-catenin, COX-2, D-DT, and α-tubulin. Densitometry of COX-2/tubulin is shown. All data are representative of 2 independent experiments.

delivery system. The rationale was that prostaglandin E_2 (PGE_2), the primary product of COX-2–mediated arachidonic acid metabolism, is an important physiologic regulator of β-catenin stabilization in CRCs (29). Consistent with this and other studies showing a role for COX-2/PGE_2 in promoting β-catenin stability (30, 31), Ad-COX-2 increased β-catenin expression in both D-DT–competent and D-DT-deficient cells (Fig. 5D). Intriguingly, we also observed a dose-dependent increase in endogenous (Fig. 5D, second panel, lower band) COX-2 when Ad-COX-2 was expressed in D-DT–competent *and* D-DT–deficient cells (Fig. 5D, second panel, upper band). Combined, these results suggest that an amplification loop exists between COX-2–derived PGE_2 and β-catenin/TCF-dependent COX-2 transcription in CRC cells and that D-DT expression is necessary for maximally promoting the COX-2 expression component of this pathway (Fig. 6).

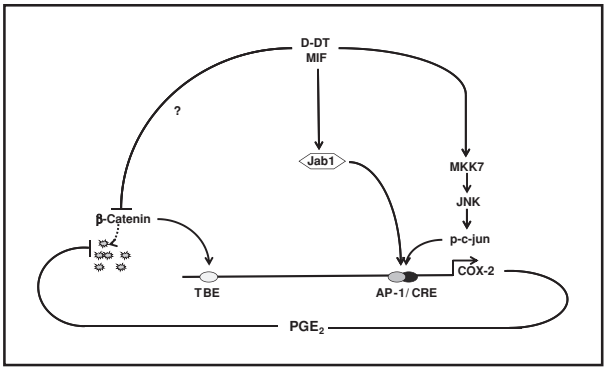

Figure 6. Proposed scheme of D-DT–mediated COX-2 expression in CRC cell lines. TBE, TCF-binding element.

Discussion

Prior studies from our laboratory revealed that MIF and D-DT cooperatively regulate JNK activation leading to AP-1–mediated VEGF and CXCL-8 expression (19). The combined effects of MIF and D-DT on angiogenic growth factor expression was found to provide nearly 90% of the neoangiogenic potential of those lung adenocarcinoma cell lines tested when assessed by human umbilical vein endothelial cell (HUVEC) tube formation and migration assays (19). Importantly, the additive effects of MIF and D-DT in NSCLC were dependent upon the MIF and D-DT cell surface receptor CD74 (19). Although not shown here, both HT-29 and HCT-116 cells used in this study express

CD74 at moderately high levels consistent with prior studies showing elevated CD74 expression in a large percentage (~85%) of human colorectal adenomas (32). Because COX-2 is an important transcriptional target of MIF (12, 13) and is phenotypically important for CRC maintenance and progression (33–35), we sought to determine whether D-DT and MIF similarly regulate COX-2 expression in colorectal adenocarcinoma cells. We now show that, like MIF, D-DT is both sufficient and necessary for maximal COX-2 expression in CRC cell lines. Importantly, our results indicate, for the first time, an important regulatory role for D-DT in controlling the stability and transcriptional activity of β-catenin. Although studies are currently underway to elucidate this pathway in more detail and to validate these findings in a mouse model of spontaneously arising CRC (36), our current results indicate that the loss of β-catenin stabilization in D-DT–deficient cells is overcome by ectopic reintroduction of COX-2. Coinciding with the apparent rescue of β-catenin expression in Ad-COX-2–expressing D-DT–deficient cells is the rescue of endogenous COX-2, indicating the existence of an amplification loop in these cells. This is telling because other rescue attempts, including overexpressing both wild-type and phosphorylation mutants of β-catenin (Fig. 2C and data not shown), CSN5 (data not shown) and CA MKK7 (Fig. 4C), were all unsuccessful at restoring endogenous COX-2 expression. The lack of success with these rescue attempts was not surprising, given the fact that both β-catenin– and MKK7-induced AP-1 activation seem to require D-DT for maximal activities (β-catenin through enhanced stabilization and MKK7/AP-1 through regulation of CSN5). Given the results, we hypothesize that rescue of endogenous COX-2 by Ad-COX-2 is through PGE_2-mediated restabilization of β-catenin and subsequent β-catenin/TCF-mediated COX-2 transcription. This hypothesis further predicts that the loss of β-catenin stabilization observed in D-DT deficiency (Fig. 2) is due either to 1) the loss of COX-2/PGE_2 because of defective JNK/c-jun/AP-1 activity (Figs. 4 and 5) or 2) D-DT–dependent regulation of β-catenin stability functions through the same pathway as that of PGE_2. Although the data suggest that Ad-COX-2–mediated β-catenin stabilization is responsible for the rescue of endogenous COX-2 in D-DT–deficient cells, we cannot rule out the possibility that PGE_2 also activates c-jun phosphorylation as has been suggested (37, 38). Recent studies reveal that PGE_2 is an important endogenous regulator of β-catenin stabilization and Wnt signaling/transcription (29, 30). Studies are currently underway to evaluate the relative contribution of PGE_2 to D-DT–dependent β-catenin stabilization. If our data do reveal a role for PGE_2 in β-catenin stabilization in these cells, this would suggest that D-DT participates in an amplification loop consisting of β-catenin–dependent COX-2 transcription and COX-2–dependent β-catenin stabilization (Fig. 6). Nevertheless, our current results indicate that D-DT, and likely MIF, is necessary for maximal signaling/transcription through this pathway.

The fact that MIF family members facilitate both JNK/c-jun pathway activation and β-catenin stabilization suggests that COX-2 may be only one of a number of important gene products associated with colorectal carcinoma disease maintenance and progression. Studies by the Behrens' group show that these 2 pathways synergize to enhance intestinal tumorigenesis (39, 40). Specifically, JNK-phosphorylated c-jun interacts with β-catenin/TCF4 complexes on the promoters of Wnt and AP-1 transcriptional target genes and disruption of either pathway dramatically alters the tumor burden and the transcription of these target genes tumors from the Apc^{Min} mouse model of intestinal tumorigenesis (39, 40). Given the apparent contributions of D-DT (and likely MIF) to both JNK/c-jun activation and β-catenin stabilization, it is reasonable to speculate that these MIF family members may promote CRC pathogenesis through this pathway.

Consistent with an important contributing role for MIF family members to intestinal neoplastic disease progression and pathogenesis, genetic ablation or small molecule inhibition of MIF alone results in significant reductions in intestinal tumorigenesis in mouse models of CRC (41, 42). Our results, coupled with earlier findings showing potent additive effects of MIF and D-DT in contributing to NSCLC angiogenesis (19), strongly suggest that the dual targeting of both MIF family members simultaneously may represent a potent and novel anticancer, immune, or inflammatory disorder chemotherapeutic targeting strategy. Finally, because of the overlapping nature of biological activities between MIF and D-DT and the nonphysiologic activity of the catalysis for which D-DT was named (D-dopachrome tautomerase), we propose changing the name of D-DT to MIF-2.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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