Regulation of *CYP24* splicing by 1,25-dihydroxyvitamin D₃ in human colon cancer cells

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Abstract

CYP24 is a well-established vitamin D receptor (VDR) target gene. The active VDR ligand 1,25(OH)₂D₃ regulates its own catabolism by increasing *CYP24* expression. It is well known that in the presence of 1,25(OH)₂D₃, VDR binds to VDREs in the promoter region of *CYP24* and initiates *CYP24* transcription. However, little is known about the role of 1,25(OH)₂D₃ in the posttranscriptional modulation of *CYP24*. In this study, we investigated the functional significance of 1,25(OH)₂D₃ in *CYP24* RNA splicing in colon cancer cells. Using RT-PCR, we found that 1,25(OH)₂D₃ actively induces *CYP24* splicing in a time-dependent manner and *CYP24* splicing pattern could be cell type or tissue specific. The induction of RNA splicing by 1,25(OH)₂D₃ was mainly *CYP24* selective. Treatment of cells

with parathyroid hormone inhibited basal *CYP24* splicing, but failed to inhibit 1,25(OH)₂D₃-induced *CYP24* splicing. Further experiments demonstrated that new RNA synthesis was required for the induction of *CYP24* splicing by vitamin D. In addition, alteration of multiple signaling pathways also affected *CYP24* splicing and cellular sensitivity in response to vitamin D appeared to correlate with the induction of *CYP24* splicing. These results suggest that 1,25(OH)₂D₃ not only regulates *CYP24* transcription, but also plays an important role in posttranscriptional modulation of *CYP24* by inducing its splicing. Our findings reveal an additional regulatory step that makes the vitamin D mediated action more prompt and efficient.

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Introduction

Alternative splicing in relation to cancer has been well investigated (Chen & Manley 2009, Nilsen & Graveley 2010). However, the general RNA splicing (the conversion of precursor-mRNA to functional-mRNA) that makes a gene functional in relation to normal or cancer cells is rarely investigated. It is generally thought that once RNA is transcribed, the immature (or unspliced) RNA is not stable and quickly spliced to mature RNA. Pre-mRNA splicing, the joining of two exons accompanied by the removal of intronic sequence, requires a very large ribonucleoprotein (RNP) complex termed the spliceosome (Wahl *et al.* 2009). The spliceosome must be guided to the correct sites for splicing. Accumulating evidence suggests that RNA splicing is coupled with transcription activation through a subset of nuclear receptor coregulators (Monsalve *et al.* 2000, Auboeuf *et al.* 2002, 2004, 2005, 2007). One of the initial steps in the splicing process is the recognition of the 5' splicing site (SS), which is accompanied in part by the formation of U1 snRNP and the base pairing of the U1 snRNA with the 5' SS sequence. This early U1–5' SS duplex aids the assembly of the 'pre-spliceosome' on the transcript and is then unwound to

allow the formation of the U6–5' SS duplex, leading to the creation of the 'active' spliceosome. Interestingly, these two events require proteins that can also act as transcriptional coregulators (Auboeuf *et al.* 2005). One of these coregulators is NCoA62/Skip, which could interact with vitamin D receptor (VDR) and regulate vitamin D-mediated transcription and splicing (Zhang *et al.* 2003). It is reported that a dominant negative inhibitor of NcoA62/Skip interferes with appropriate splicing of 1,25(OH)₂D₃-induced transcripts derived from a GH minigene cassette, in which the human GH gene was under the control of the thymidine kinase promoter and four copies of a VDRE from the rat osteocalcin gene (Zhang *et al.* 2003). These data suggest a potential role for NcoA62/Skip in coupling VDR-mediated transcription to RNA splicing.

Through analysis of *CYP24* expression in colon cancer cells using quantitative RT-PCR, we found that different primer sets generated significantly different results in response to vitamin D treatment. This finding prompted us to further carefully investigate whether the active hormone 1,25(OH)₂D₃ regulates endogenous *CYP24* RNA splicing in addition to the transcriptional activation in colon cancer cells and whether the splicing pattern could be cell type

specific. *CYP24* is the direct target gene of vitamin D, its protein product is 1,25-dihydroxyvitamin D₃ 24-hydroxylase, which is the catabolic enzyme of both 1,25(OH)₂D₃ and 25(OH)D₃. Therefore, the regulation of *CYP24* expression is critical for both the protective effects of vitamin D against carcinogenesis and catabolism of vitamin D.

Materials and Methods

Hormones and chemical reagents

1,25(OH)₂D₃ and kinase inhibitors (SB203580 (p38MAPKi) and SP600125 (JNKi)) were purchased from EMD Biosciences (La Jolla, CA, USA). TPA was supplied from the NCI repository (Frederick, MD, USA). Theophylline, actinomycin D, and cycloheximide were purchased from Sigma. 1,25(OH)₂D₃ was dissolved in ethanol and stored at -80°C . Parathyroid hormone (PTH, bovine, 1–34) was purchased from AnaSpec, Inc. (San Jose, CA, USA) and dissolved in PBS. Other reagents were dissolved in DMSO and stored at -20°C .

Cell culture, cell proliferation and biopsy samples

The colon cancer cell lines HT29, Caco-2, and HCT116 were obtained from ATCC (Manassas, VA, USA). The cell lines were maintained and cultured in RPMI 1640 media (Invitrogen) supplemented with 10% fetal bovine serum (Atlanta Biologicals, Lawrenceville, GA, USA), 2×10^{-4} mol/l L-glutamine and 1% antibiotic-antimycotic solution (Invitrogen). All cells were treated with hormones or reagents at selected concentrations in 60 mm culture dishes. 1,25(OH)₂D₃ and PTH were used at concentration of 10^{-8} and 6×10^{-8} mol/l respectively. The concentrations of other reagents were determined by preliminary experiments in our laboratory and from reports in the literature. Cell proliferation was determined by crystal violet assay as described previously (Peng *et al.* 2010). Biopsy samples were collected from high-risk colon cancer patients during colonoscopy at University of Illinois hospital. Patients' consents and appropriate approvals for the studies described in this report were obtained in advance. Fresh biopsy samples were quickly frozen in liquid nitrogen and stored at -80°C until RNA isolation.

RNA isolation and RT-PCR

Total RNA extraction, RT and real-time PCRs were performed as described previously (Peng *et al.* 2004). RNA was further subjected to DNase I (Ambion, Austin, TX, USA) digestion and purification using RNeasy Mini kit (Qiagen) before reverse transcription. The cycling conditions for PCR were 35 cycles of 20 s at 95°C , 20 s at 60°C , and 30 s at 72°C . The cycling conditions for real-time PCR were: 40 cycles of 15 s at 95°C , 15 s at 60°C , and 20 s at

72°C . Real-time PCR product quality was monitored using post-PCR melt curve analysis. GAPDH was amplified for 24 cycles using conventional PCR or using real-time PCR from the same cDNA samples as a housekeeping gene for normalization. All primers were designed and ordered from Integrated DNA Technologies (Coralville, IA, USA). Table 1 lists all the specific primers used in this study. At least two independent experiments of RT-PCR were carried out to confirm the consistency of the data and the representative data are presented.

Immunoblot analysis and immunofluorescent staining

HT29 cell lysates were prepared after 24 h treatment with 10^{-8} mol/l 1,25(OH)₂D₃ and subjected to immunoblot analysis (Peng *et al.* 2004). Procedures for immunostaining and confocal microscopy were described previously (Peng *et al.* 2006). *CYP24* (vitamin D₃ 24-hydroxylase) mouse monoclonal antibody for immunoblotting and immunofluorescent staining was purchased from Abnova Taiwan Corp. (Taipei, Taiwan).

Results

CYP24 splicing in colon cancer cells and human colon tissue samples

To determine the effect of vitamin D on *CYP24* splicing in colon cancer cells, we designed specific primers located in exons 1 and 2 of *CYP24* gene. Since intron 1 is a small intron (200 bp; Table 1), this pair of primers allowed us to detect both spliced and unspliced *CYP24* RNA using RT-PCR. As shown in Fig. 1A, different cell lines exhibited differential *CYP24* splicing pattern in the absence of vitamin D treatment. In colon cancer cell lines HT29 and Caco-2, the basal *CYP24* RNA remained unspliced or partially spliced, respectively, whereas in HCT116 cells, *CYP24* RNA was observed as already spliced; no unspliced *CYP24* was observed. 1,25(OH)₂D₃ significantly induced *CYP24* splicing in HT29 and Caco-2 cells, suggesting that in addition to transcriptional activation of *CYP24*, 1,25(OH)₂D₃ also regulates the maturity of *CYP24* RNA in these cells. Since basal *CYP24* splicing could be cell type specific, we further examined human colon tissue samples directly obtained from 22 patients. As shown in Fig. 1B, five *CYP24* splicing patterns were identified, 11 of the 22 patients displayed pattern 1 (in colon aberrant crypt foci (ACF), both unspliced and spliced *CYP24* RNA was detectable, however only unspliced *CYP24* RNA was detectable in the adjacent normal colon tissue); 2 out of 22 patients displayed pattern 5 (*CYP24* remained spliced in both ACF and the adjacent normal tissue samples). Overall, in 15 out of 22 ACF samples (68%), *CYP24* remained spliced or partially spliced; whereas in only 8 out of 22 normal colon tissue samples (36%), *CYP24* remained spliced or partially spliced; in 64% of normal colon tissue

Table 1 Primers used for gene splicing analysis. GAPDH primers were used for both real-time PCR and conventional PCR, all other primers were used for conventional PCR. CYP24Ex1-2 primer pair was the major primer pair used for CYP24 splicing pattern analysis unless indicated

Primer names	Locations	Primer sequences (5'–3')	Spliced (bp)	Unspliced (bp)
CYP24 Ex1-2	F (exon 1)	TCA AGA AAC AGC ACG ACA CC	200	400
	R (exon 2)	CGT AGC CTT CTT TGC GGT AG		
CYP24 Ex10-11	F (exon 10)	GGT GTT GGG ATC CAG TGA AG	204	302
	R (exon 11)	GTG GCC TGG ATG TCG TAT TT		
CYP27B1	F (exon 5)	CACCTGAACCACTTCCTGTT	132	335
	R (exon 6)	CCGGGAGAGCTCATACAGAG		
VDR	F (exon 6)	GCAGAGGAAGCGGGAGATGACCT	189	443
	R (exon 7)	CCACCATCATTCACACGAAC		
IGFBP5	F (exon 2)	GAGCTGAAGGCTGAAGCAGT	237	855
	R (exon 3)	GAATCCTTTGCGGTCACAAT		
GAPDH	F	ATC ACT GCC ACC CAG AAG AC	133	133
	R	TTT AGC TCA GGG ATG ACC TT		

F, Forward; R, reverse

samples, CYP24 remained unspliced. These results suggest that CYP24 may be a potential oncogene (Horváth *et al.* 2010) and demonstrate that the regulation of CYP24 splicing is a common phenomenon.

Characterization of vitamin D-induced CYP24 splicing

Since 1,25(OH)₂D₃ induced CYP24 splicing in colon cancer cells, we further characterized vitamin D-induced CYP24 splicing and expression using HT29 cells. As shown in Fig. 2A, 1,25(OH)₂D₃ induced CYP24 splicing in a time-dependent manner. CYP24 splicing started to occur after 45 min of 1,25(OH)₂D₃ treatment; after 120 min of treatment, most of the CYP24 RNA was spliced, suggesting that CYP24 splicing regulation by 1,25(OH)₂D₃ was very prompt and efficient. To determine that the vitamin D-induced CYP24 splicing was not alternative splicing, we designed another pair of primers located in exons 10 and 11, because intron 10 is also a small intron (98 bp), the splicing at intron 10 can also easily be detected by RT-PCR. Figure 2B shows the CYP24 splicing at different introns after 1,25(OH)₂D₃ treatment for 2 h. At intron 1, the splicing appeared to be more efficient than the splicing at intron 10 in the presence of 1,25(OH)₂D₃. These results also suggest that the vitamin D-induced CYP24 splicing is not alternative splicing, but the maturity of CYP24 RNA; which was confirmed by CYP24 protein (24-hydroxylase) expression analyzed by immunoblotting (Fig. 2C). The treatment with 1,25(OH)₂D₃ induced CYP24 protein (55 kDa) expression, which is associated with the expression of mature (spliced) CYP24 mRNA. In addition, consistent results were obtained by immunofluorescent staining of 24-hydroxylase in cultured HT29 cells, 24-hydroxylase was induced by vitamin D treatment and it was localized in the perinuclear region (data not shown).

Specificity of RNA splicing regulation by vitamin D

Since 1,25(OH)₂D₃ induced CYP24 splicing and CYP24 is an immediate vitamin D target gene, we examined whether it

regulates the splicing of other vitamin D responsive genes. Several vitamin D responsive genes including CYP27B1 (Turunen *et al.* 2007), VDR (Hussain-Hakimjee & Mehta 2009), and IGFBP5 (Matilainen *et al.* 2005) were selected for splicing detection keeping CYP24 as a positive control. Figure 3 shows the RT-PCR analysis of the splicing pattern of these genes after 1,25(OH)₂D₃ treatment of HT29 and HCT116 cells. 1,25(OH)₂D₃ induced CYP24 splicing as expected, slightly attenuated CYP27B1 splicing and had no significant effect on VDR and IGFBP5 splicing in HT29 cells. In HCT116 cells, all the examined genes remained spliced; 1,25(OH)₂D₃ increased the expression of the spliced CYP24 and had little effect on CYP27B1 and VDR expression, IGFBP5 was barely detectable in HCT116 cells. These results suggest that splicing regulation by vitamin D might be

Figure 1 CYP24 splicing in colon cancer cells and human tissue samples. (A) CYP24 splicing pattern in the absence and presence of vitamin D in colon cancer cells. Three colon cancer cell lines were treated with ethanol (control, C) and 10⁻⁸ mol/l 1,25(OH)₂D₃ (D3) for 4 h and subjected to RT-PCR analysis. Close C_T from all the cDNA samples were obtained by real-time PCR analysis of GAPDH expression. 1,25(OH)₂D₃ induced CYP24 splicing in HT29 and Caco-2 cells. (B) CYP24 splicing patterns identified in 22 pairs of human tissue samples. Aberrant crypt foci (A) and adjacent normal colon tissues (N) displayed different CYP24 splicing pattern. The number under each pattern represents the ratio of the selected pattern and in the total number of samples. M, DNA maker. Equal C_T from both tissue samples were obtained by real-time PCR analysis of 18S expression.

Figure 2 Effect of $1,25(\text{OH})_2\text{D}_3$ treatment on *CYP24* splicing and protein expression in HT29 cells. (A) HT29 cells were treated with 10^{-8} mol/l $1,25(\text{OH})_2\text{D}_3$ for 30, 45, 60, and 120 min, *CYP24* splicing was examined by RT-PCR. $1,25(\text{OH})_2\text{D}_3$ (D_3) treatment induced *CYP24* splicing in a time-dependent manner. GAPDH served as a loading control. (B) *CYP24* splicing at different introns after $1,25(\text{OH})_2\text{D}_3$ treatment for 2 h. At intron 1, the splicing appeared to be more efficient than the splicing at intron 10 in the presence of $1,25(\text{OH})_2\text{D}_3$. (C) Immunoblot analysis of *CYP24* protein expression in HT29 cells after $1,25(\text{OH})_2\text{D}_3$ treatment for 24 h. $1,25(\text{OH})_2\text{D}_3$ also induced *CYP24* protein expression, which is associated with the expression of mature (spliced) *CYP24* mRNA. M, protein markers showing standard molecular weight; C, control; D_3 , $1,25(\text{OH})_2\text{D}_3$.

gene or cell specific, although we cannot exclude that vitamin D could also regulate the splicing of other target genes. It is interesting to note that we could not detect spliced *IGFBP5* in HT29 cells regardless of vitamin D treatment.

*Effect on PTH on $1,25(\text{OH})_2\text{D}_3$ -induced *CYP24* splicing in colon cancer cells*

The functional significance of PTH has been closely correlated with vitamin D action (Welsh *et al.* 1991, Murayama *et al.* 1999). PTH has been shown to decrease *CYP24* expression levels by altering its mRNA stability in AOK-B50 (porcine kidney proximal tubule) cells (Zierold *et al.* 2001, 2003). Experiments were designed to examine whether PTH inhibits vitamin D-induced *CYP24* splicing. As shown in Fig. 4, PTH at 6×10^{-8} mol/l significantly inhibited the basal *CYP24* splicing, especially in HCT116 cells, however, it failed to suppress $1,25(\text{OH})_2\text{D}_3$ induced *CYP24* splicing. Interestingly, PTH appeared to increase the

accumulation of unspliced *CYP24* RNA in both HT29 and HCT116 cells in the absence of $1,25(\text{OH})_2\text{D}_3$; in the presence of $1,25(\text{OH})_2\text{D}_3$, PTH enhanced the expression of spliced *CYP24* in HCT116 cells.

*Effect of RNA and protein synthesis inhibitors on vitamin D-induced *CYP24* splicing*

To determine whether new RNA or protein synthesis is required for $1,25(\text{OH})_2\text{D}_3$ -induced *CYP24* splicing, HT29 cells were pretreated with actinomycin D or cycloheximide for 1 h to inhibit RNA or protein synthesis, then the cells were treated with $1,25(\text{OH})_2\text{D}_3$ for 1 h to induce *CYP24* splicing. As shown in Fig. 5, actinomycin D treatment completely blocked $1,25(\text{OH})_2\text{D}_3$ -induced *CYP24* splicing. When the cells were incubated with cycloheximide and vitamin D together there was a reduction in the spliced *CYP24* compared with the splicing induced by vitamin D only in the absence of cycloheximide, indicating that cycloheximide only inhibited $1,25(\text{OH})_2\text{D}_3$ -induced splicing. These results demonstrate that new RNA synthesis, instead of new protein synthesis, may be required for vitamin D-induced *CYP24* splicing. Surprisingly, in the presence of cycloheximide, the unspliced *CYP24* appeared to be accumulating in comparison to the control cells (lane 1) in HT29 cell line.

*Effect of signaling alterations on $1,25(\text{OH})_2\text{D}_3$ -induced *CYP24* splicing in HT29 cells*

It is well established that multiple signaling pathways are regulated by vitamin D treatment or involved in vitamin D action. It is therefore of interest to determine whether these signaling alterations affect vitamin D-induced *CYP24* splicing. We selected the signaling pathways that have been reported to be involved in vitamin D action including PKA (Avila *et al.* 2007, Khanal *et al.* 2008), PKC (Wang *et al.* 2010, Buitrago *et al.* 2011), p38 MAP kinase (Pardo *et al.* 2006, Brosseau *et al.* 2010, Buitrago *et al.* 2011), and c-Jun N-terminal protein kinase (JNK) pathway (Chen *et al.* 1999, Qi *et al.* 2002, Brosseau *et al.* 2010) to examine how activation or inhibition of these signaling pathways would affect vitamin D-induced *CYP24* splicing. As shown in Fig. 6, activation of PKA in HT29 cells by theophylline induced *CYP24* splicing, but appeared to inhibit *CYP24* transcription (Fig. 6A) and had no significant effect on $1,25(\text{OH})_2\text{D}_3$ -induced *CYP24* splicing; whereas PKC activation appeared to attenuate both basal- and vitamin D-induced *CYP24* splicing. Inhibition of p38 MAP kinase with SB203580 completely inhibited both transcription and splicing in the absence of $1,25(\text{OH})_2\text{D}_3$, but failed to suppress *CYP24* splicing in the presence of $1,25(\text{OH})_2\text{D}_3$. Inhibition of JNK MAP kinase pathway with SP600125 induced *CYP24* splicing in the absence of $1,25(\text{OH})_2\text{D}_3$ treatment and also further enhanced $1,25(\text{OH})_2\text{D}_3$ -induced *CYP24* splicing. These results indicate that *CYP24* splicing can be affected by multiple signaling pathways and vitamin D could also induce *CYP24* splicing by affecting these signaling pathways.

Figure 3 Differential effect of $1,25(\text{OH})_2\text{D}_3$ on the splicing of mRNA precursors of vitamin D responsive genes in HT29 and HCT116 cells. HT29 and HCT116 cells were treated for 2 h with $1,25(\text{OH})_2\text{D}_3$ and then subject to RT-PCR analysis. Equal C_T from the cDNA samples were obtained by real-time PCR analysis of GAPDH expression. $1,25(\text{OH})_2\text{D}_3$ induced *CYP24* splicing, slightly attenuated *CYP27B1* splicing and had no effect on *VDR* and *IGFBP5* splicing in HT29 cells; in HCT116 cells, most of the RNA remained spliced and vitamin D had little effect on *CYP27B1* and *VDR*, but increased *CYP24* mRNA expression.

CYP24 splicing pattern is correlated with cellular sensitivity in response to vitamin D in multiple colon cancer cell lines

We further evaluated *CYP24* splicing pattern and cell proliferation in response to vitamin D in multiple colon cancer cell lines side by side. As shown in Fig. 7, $1,25(\text{OH})_2\text{D}_3$ treatment significantly inhibited cell proliferation by 42% ($P < 0.01$, $n = 8$) in HT29 cells and by 26% ($P < 0.01$, $n = 8$) in Caco-2 cells. In these vitamin D sensitive cell lines, *CYP24* remained unspliced or partially spliced in the absence of $1,25(\text{OH})_2\text{D}_3$, inhibition of cell proliferation by vitamin D treatment was accompanied by significant induction of *CYP24* splicing. On the other hand, $1,25(\text{OH})_2\text{D}_3$ treatment had little or minor inhibitory effect on cell proliferation in HCT116, SW480, and LS174 cells, where *CYP24* remained spliced or largely spliced even in the absence of $1,25(\text{OH})_2\text{D}_3$, vitamin D treatment had little effect on *CYP24* splicing pattern in these cell lines. These results suggest that cellular sensitivity in response to vitamin D might be correlated to *CYP24* splicing pattern.

Discussion

In cancer biology, each regulatory point in the control of gene expression (which includes chromatin structure, splicing and polyadenylation of mRNA precursors, translation, and mRNA and protein stability) is subject to profound

alterations during the development of most, if not all, cancers (Venables 2006, Mayr & Bartel 2009, Chi *et al.* 2010, Silvera *et al.* 2010). Understanding of these alterations is important for both prevention and cancer treatment. Genome-wide approaches have revealed that tumorigenesis often involves large-scale alterations in alternative splicing (Venables *et al.* 2009); therefore, alternative splicing is well investigated in relation to cancer. The maturation of mRNA precursors (or RNA splicing), although widely reported in the literature, has not been associated with cancer or has not been investigated in response to cancer preventive or therapeutic agents.

We found that in colon cancer cells, *CYP24* RNA splicing can be actively regulated by vitamin D treatment and the splicing pattern could be cell type specific. In addition, RNA splicing patterns in the tested cell lines were associated with cellular sensitivity in response to vitamin D. In human tissue samples, *CYP24* splicing was also observed: in 68% of the examined ACF samples, *CYP24* remained spliced or partially spliced; whereas in only 36% of the examined normal colon tissue samples, *CYP24* remained spliced or partially spliced. However, whether the *CYP24* splicing pattern is of any diagnostic significance for ACF detection is still not clear. Analysis of a large amount of samples is needed for this correlation. Time-course studies demonstrate that the regulation of *CYP24* splicing by vitamin D is very prompt and efficient; however, it appears that there was a reduction in the efficiency of splicing at intron 10 compared with intron 1. It is possible that the reduction in the splicing efficiency between introns 1 and 10 may be dependent on the distance between the two introns. The reduced efficiency of gene splicing based on the position of introns was also reported previously in human neuroblastoma and fibrosarcoma cell lines (Jarnail & Richard 2009). Among the genes tested for splicing regulation by vitamin D, *CYP24* seemed to be the major target gene, suggesting that regulation of splicing by vitamin D could be gene selective. However, we cannot rule out that the splicing of other vitamin D target genes might also be regulated by vitamin D. It should be noted that the transcription of *VDR* and *IGFBP5* was not significantly regulated by vitamin D in our experimental setting and whether splicing was coupled with transcription activation or not still needs to be determined, although the experiment

Figure 4 Effect of PTH on $1,25(\text{OH})_2\text{D}_3$ -induced *CYP24* splicing in colon cancer cells. HT29 and HCT116 cells were treated with $1,25(\text{OH})_2\text{D}_3$ (10^{-8} mol/l) or PTH (6×10^{-8} mol/l), respectively, or in combination for 2 h, *CYP24* splicing pattern was analyzed by RT-PCR. M, DNA marker. Very close C_T from all the cDNA samples were obtained by real-time PCR analysis of GAPDH expression. PTH inhibited basal *CYP24* splicing in both cell lines, but failed to inhibit $1,25(\text{OH})_2\text{D}_3$ -induced *CYP24* splicing.

Figure 5 RNA synthesis is required for 1,25(OH)₂D₃-induced CYP24 splicing in HT29 cells. HT29 cells were pretreated with actinomycin D (Act-D, 10⁻⁵ g/l) or cycloheximide (CHX, 10⁻⁵ mol/l) for 1 h, then treated with 1,25(OH)₂D₃ for 1 h, CYP24 splicing was analyzed by RT-PCR. GAPDH served as loading control. Actinomycin D treatment blocked 1,25(OH)₂D₃-induced CYP24 splicing, but cycloheximide did not fully block 1,25(OH)₂D₃-induced splicing.

with actinomycin D suggests that new RNA synthesis is needed for vitamin D mediated CYP24 splicing.

The role of PTH has been well established for modulating vitamin D action. In HT29 and HCT116 colon cancer cells the appearance of parathyroid nuclear G-protein coupled receptor is responsible for a direct nuclear action for PTH (Shen *et al.* 2007). We observed that PTH inhibited the splicing of CYP24, however this suppression of CYP24 splicing was overcome in the presence of 1,25(OH)₂D₃. These results suggested that in regulation of CYP24 splicing, vitamin D may have a selective preference for splicing of CYP24 over PTH, although the detailed mechanism for this selective action of vitamin D and PTH is not currently known. It should be noted that this is the first report of PTH inhibiting CYP24 RNA splicing. It was previously reported that PTH diminishes the stability of CYP24 mRNA to decrease the expression of CYP24 mRNA in AOK-B50 cells (Zierold *et al.* 2001, 2003). Our finding demonstrates another mechanism of PTH action in decreasing CYP24 mRNA expression: PTH decreases CYP24 mRNA expression by inhibiting CYP24 splicing. As for the interaction between PTH and vitamin D in regulation of CYP24, it was shown that PTH and vitamin D synergistically increases CYP24 mRNA expression in UMR106 osteoblastic cells (Armbrecht *et al.* 1998), which is consistent with our observation (Fig. 4). Although PTH inhibited basal CYP24 splicing in HT29 and HCT116 cells, it appeared to enhance the CYP24 transcription, since the transcripts were not spliced, this effect might not be detected by northern blot analysis.

Our experiments with signaling alteration indicate that multiple signaling pathways are involved in splicing regulation. Theophylline is a competitive non-selective phosphodiesterase inhibitor (Essayan 2001), which raises intracellular cAMP, activates PKA and inhibits TNF-α (Marques *et al.* 1999, Deree *et al.* 2008). Theophylline induced CYP24 splicing in HT29 cells, however it also inhibited CYP24 transcription; whereas vitamin D stimulated

both CYP24 transcription and splicing. Therefore, although vitamin D was reported to activate PKA (Avila *et al.* 2007, Khanal *et al.* 2008), whether vitamin D induces CYP24 splicing through PKA still needs further investigation. Vitamin D was also reported to activate PKC (Chen *et al.* 2010, Wang *et al.* 2010, Centeno *et al.* 2011), however, vitamin D apparently did not induce CYP24 splicing through activation of PKC, because activation of PKC attenuated CYP24 splicing. Inhibition of p38 MAPK inhibited both CYP24 transcription and splicing, but failed to block vitamin D-induced splicing, suggesting p38 MAPK might not be involved in vitamin D mediated CYP24 splicing.

Figure 6 Effect of multiple signaling alterations on 1,25(OH)₂D₃-induced CYP24 splicing in HT29 cells. Cells were treated with theophylline (Theo, 10⁻⁴ mol/l, PKA activator) (A), TPA (5 × 10⁻⁸ mol/l, PKC activator) (B), p38 MAP kinase inhibitor (p38-i, SB203580, 2.5 × 10⁻⁵ mol/l) (C), and JNK inhibitor (JNKi, SP600125, 3 × 10⁻⁵ mol/l) (D), respectively, or in combination with 1,25(OH)₂D₃ (10⁻⁸ mol/l) for 2 h and CYP24 splicing pattern was examined by RT-PCR to determine how these signaling alterations affect vitamin D induced CYP24 splicing. Close C_T from all the cDNA samples were obtained by real-time PCR analysis of GAPDH expression. Note both theophylline and JNK inhibitor induced CYP24 splicing.

Figure 7 Correlation of induction of *CYP24* splicing with cellular sensitivity in response to vitamin D in multiple colon cancer cell lines. (A) Splicing pattern of *CYP24* RNA in HT29, Caco-2, HCT116, SW480, and LS174 in the absence and presence of 1,25(OH)₂D₃ (2 h treatment) as examined by RT-PCR. (B) Effect of 1,25(OH)₂D₃ on cell proliferation in HT29, Caco-2, HCT116, SW480, and LS174 cells. Cells were treated with 10⁻⁸ mol/l 1,25(OH)₂D₃ for 4 days and cell proliferation was evaluated using Crystal Violet assay. Data are expressed as mean ± s.d., n=8, **P<0.01 (Student's *t*-test).

Interestingly, treatment of HT29 cells with a JNK inhibitor – SP600125 showed similar effect to vitamin D on *CYP24* splicing, a question therefore arises: whether vitamin D induces *CYP24* splicing by inhibiting JNK signaling. Reports in the literature about the interaction between JNK and vitamin D do not support this concept. Yasunami *et al.* (2004) demonstrated that SP600125 dose-dependently inhibited the *CYP3A4* promoter activity induced by 1,25(OH)₂D₃; however, SP600125 did not affect 1,25(OH)₂D₃-induced transactivation of the DR3 via VDR. Buitrago *et al.* (2011) showed that 1,25(OH)₂D₃ promotes the phosphorylation of JNK 1/2, the response was fast and maximal phosphorylation of the enzyme was observed at physiological doses of 1,25(OH)₂D₃. Kondo *et al.* (2004) showed that 1,25(OH)₂D₃ treatment of ST2 cells decreased the amount of phosphorylated c-Jun protein (phospho-c-Jun), while the total amount of c-Jun remained constant and the amount of phosphorylated JNK was nearly unchanged by vitamin D treatment. These reports suggest that vitamin D might not inhibit JNK activity, although the interaction between JNK and vitamin D could be cell type or gene specific. Overall, our results suggest that 1,25(OH)₂D₃-induced *CYP24* splicing might involve JNK inhibition.

Previous study showed that vitamin D also induces *CYP24* splicing in LNCaP prostate cancer cells (Muindi *et al.* 2007). However, the mechanism of vitamin D mediated *CYP24* splicing remains to be established. In this study, we showed that 1,25(OH)₂D₃ quickly triggered *CYP24* splicing in colon cancer cells, which might not require new protein synthesis, we therefore designed experiments by alteration of signaling

transduction to examine whether the non-genomic signaling pathway of vitamin D mediates *CYP24* splicing. As discussed above, the results were still inconclusive since alteration of multiple signaling pathways was found to affect *CYP24* splicing. Although the genomic pathway of vitamin D in inducing *CYP24* splicing was not explored in our study, it is possible that 1,25(OH)₂D₃, through binding to VDR, interacts with some components of the spliceosome, such as NCoA62/Ski (Zhang *et al.* 2003) to trigger the assembling of spliceosome to initiate splicing; however, how *CYP24* is specifically selected for vitamin D-induced splicing remains to be a question.

The correlation studies regarding *CYP24* splicing induction and cellular sensitivity suggest that the basal level of mature *CYP24* might contribute to cellular resistance in response to vitamin D in colon cancer cells. Cells with a high basal level of mature *CYP24* could be resistant to vitamin D treatment, since the 24-hydroxylase is immediately available for catabolizing vitamin D. However, sensitive cells such as HT29 and Caco-2 cells express low level of mature *CYP24*, upon vitamin D treatment, both significant *CYP24* induction and inhibition of cell proliferation can be observed in these sensitive cell lines. These data indicate that in sensitive cell lines, while the 24-hydroxylase is not immediately available, genes related to the antiproliferative effect of vitamin D could be activated upon vitamin D treatment.

In summary, this report provides novel information regarding vitamin D-mediated *CYP24* splicing and possible association among *CYP24* splicing patterns, cell transformation (ACF formation) and cellular sensitivity in response to vitamin D. Our findings shed some light on whether other steroid hormones can regulate the splicing of their selective target genes. Conceptually, similar regulation of immature RNA to its functional states by other steroid hormones is a strong possibility.

Declaration of interest

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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