



**HAL**  
open science

## **A new mechanism of SOX9 action to regulate PKC expression in the intestine epithelium**

S Dupasquier, R Abdel-Samad, R I Glazer, P Bastide, P Jay, D Joubert, Vincent  
Cavallès, P Blache, C Quittau-Prévostel

### ► **To cite this version:**

S Dupasquier, R Abdel-Samad, R I Glazer, P Bastide, P Jay, et al.. A new mechanism of SOX9 action to regulate PKC expression in the intestine epithelium. *Journal of Cell Science*, 2009, 122 (13), pp.2191-2196. <10.1242/jcs.036483>. <hal-02459514>

**HAL Id: hal-02459514**

**<https://hal.science/hal-02459514v1>**

Submitted on 29 Jan 2020

**HAL** is a multi-disciplinary open access archive for the deposit and dissemination of scientific research documents, whether they are published or not. The documents may come from teaching and research institutions in France or abroad, or from public or private research centers.

L'archive ouverte pluridisciplinaire **HAL**, est destinée au dépôt et à la diffusion de documents scientifiques de niveau recherche, publiés ou non, émanant des établissements d'enseignement et de recherche français ou étrangers, des laboratoires publics ou privés.



HAL Authorization

# A new mechanism of SOX9 action to regulate PKC $\alpha$ expression in the intestine epithelium

Sébastien Dupasquier<sup>1,2,3</sup>, Rana Abdel-Samad<sup>1,2,3</sup>, Robert I. Glazer<sup>4</sup>, Pauline Bastide<sup>1,2,3</sup>, Philippe Jay<sup>1,2,3</sup>, Dominique Joubert<sup>1,2,3</sup>, Vincent Cavallès<sup>5,6,7,8</sup>, Philippe Blache<sup>1,2,3</sup> and Corinne Quittau-Prévostel<sup>1,2,3,\*</sup>

<sup>1</sup>CNRS, UMR 5203, Institut de Génomique Fonctionnelle, 34094, Montpellier, France

<sup>2</sup>INSERM, U661, 34094, Montpellier, France

<sup>3</sup>Université Montpellier 1, 2, 34094, Montpellier, France

<sup>4</sup>Department of Oncology and Lombardi Comprehensive Cancer Center, Georgetown University, Washington, DC 20007, USA

<sup>5</sup>IRCM, Institut de Recherche en Cancérologie de Montpellier, 34298, Montpellier, France

<sup>6</sup>INSERM, U896, 34298, Montpellier, France

<sup>7</sup>Université Montpellier 1, 34298, Montpellier, France

<sup>8</sup>CRLC Val d'Aurelle Paul Lamarque, 34298, Montpellier, France

\*Author for correspondence (e-mail: Corinne.Quittau-Prevostel@igf.cnrs.fr)

Accepted 27 March 2009

Journal of Cell Science 122, 2191-2196 Published by The Company of Biologists 2009

doi:10.1242/jcs.036483

## Summary

Variations of protein kinase C (PKC) expression greatly influence the proliferation-to-differentiation transition (PDT) of intestinal epithelial cells and might have an important impact on intestinal tumorigenesis. We demonstrate here that the expression of PKC $\alpha$  in proliferating intestinal epithelial cells is repressed both *in vitro* and *in vivo* by the SOX9 transcription factor. This repression does not require DNA binding of the SOX9 high-mobility group (HMG) domain but is mediated through a new mechanism of SOX9 action requiring the central

and highly conserved region of SOXE members. Because SOX9 expression is itself upregulated by Wnt-APC signaling in intestinal epithelial cells, the present study points out this transcription factor as a molecular link between the Wnt-APC pathway and PKC $\alpha$ . These results provide a potential explanation for the decrease of PKC $\alpha$  expression in colorectal cancers with constitutive activation of the Wnt-APC pathway.

Key words: Intestine, PKC $\alpha$ , SOX9, Transcription, Wnt

## Introduction

Interfering with the molecular mechanisms regulating the expression and/or activity of the effectors of proliferation-to-differentiation transition (PDT) is an attractive approach to inhibit tumor-cell growth and/or reorientate tumor cells towards a post-mitotic differentiated state.

SOX transcription factors, which contain a high-mobility group (HMG) domain, have been clearly evidenced as important regulators of the PDT during development and adult tissue renewal (Episkopou, 2005; Kanai et al., 2005; Shimizu et al., 2007; Zorn and Wells, 2007). In particular, findings from our laboratory indicate an involvement of SOX9 in the control of proliferation, differentiation and cell fate of the intestinal epithelium (Bastide et al., 2007). However, the molecular mechanisms involved in these biological processes still remain to be elucidated. Up to now, CEACAM1 is the only SOX9 direct target identified in the intestinal epithelium whose gene expression is upregulated through the binding of the SOX9 HMG domain on a sequence similar to the *in vitro* characterized consensus sequence [A/T][A/T]CAA[A/T]G (Kamachi et al., 2000; Zalzal et al., 2008). The expression of genes encoding, for example, the CDX2 cell-differentiation marker, the carcinoembryonic antigen (CEA) or the tight-junction protein claudin 7 is inversely decreased in response to an overexpression of SOX9, but it was concluded that these repressions might be indirect, i.e. mediated through the prior transcriptional activation of as-yet-unidentified repressor genes (Blache et al., 2004; Darido et al., 2008; Jay et al., 2005). Indeed, SOX9 was always found to be a transcriptional activator in a number of physiological situations (De Santa Barbara et al., 1998; Lee et al., 2004; Ng et al., 1997)

and no SOX9 DNA-binding sequence could be identified within the promoter of these genes.

*In vitro* and *in vivo* data indicate that variations in the amount of the tumor-promoting phorbol-ester receptor protein kinase C (PKC) also drastically influence the PDT and might have an important impact on intestinal tumorigenesis. Indeed, overexpression of both PKC $\beta$ 2 and PKC $\epsilon$  stimulates the proliferation of colon epithelial cells (Murray et al., 1999; Perletti et al., 1996) and a high PKC $\beta$ 2 expression level is considered as an early promoting event in colon carcinogenesis (Gokmen-Polar et al., 2001). By contrast, overexpression of PKC $\alpha$  induces cell-growth arrest and differentiation of intestinal epithelial cells, whereas opposite effects are observed with antisense oligonucleotides encoding PKC $\alpha$  (Scaglione-Sewell et al., 1998). *In vivo*, cell-growth inhibition and differentiation that occur when intestinal cells migrate from the proliferative crypt towards the differentiated and functional villus correlate with an increase in PKC $\alpha$  expression (Saxon et al., 1994), and knockout of the gene encoding PKC $\alpha$  is associated with the acquisition of the proliferative phenotype of intestinal epithelial cells (Oster and Leitges, 2006). Finally, PKC $\alpha$  is usually decreased in colorectal cancers (CRCs), suggesting a protective role of this enzyme from intestinal tumorigenesis (Kahl-Rainer et al., 1994; Suga et al., 1998).

Leontieva and Black identified two distinct pathways that are able to regulate PKC $\alpha$  contents in intestinal epithelial cells, both of which occur at the post-translational level but with no obvious link with the PDT (Leontieva and Black, 2004). The present study establishes that transcription of the gene encoding PKC $\alpha$  can be repressed both *in vitro* and *in vivo* by SOX9 in proliferating

intestinal epithelial cells and that this repression is mediated through a DNA-binding-independent transcriptional mechanism.

## Results and Discussion

### PKC $\alpha$ transcription is repressed by SOX9 in CRC cell lines

A SOX9-dependent transcriptome (<http://www.ebi.ac.uk/ArrayExpress> database, accession number E-MEXP-859) recently performed in our laboratory suggested that doxycycline-inducible FLAG-SOX9 expression was able to decrease the level of mRNA encoding PKC $\alpha$  by more than 50% in the human HT29C116E CRC cell line. As shown in Fig. 1A,B, quantitative reverse transcriptase (RT)-PCR and western blot experiments demonstrated that a 2.5-fold increase of FLAG-SOX9 expression in these cells induced a 75% decrease in the amount of mRNA encoding PKC $\alpha$  and a 50% decrease of PKC $\alpha$  protein, thus confirming a SOX9-mediated repression of PKC $\alpha$  transcription. Consistent with this result, the luciferase activity of reporter plasmids containing either the -1571

to +227 or the -227 to +77 minimum promoter of the gene encoding PKC $\alpha$  was inhibited by 40-60% in HT29C116E and HCT116 human CRC cell lines that were transiently transfected with SOX9 (Fig. 1C), demonstrating that SOX9-dependent PKC $\alpha$  repression is not restricted to HT29C116E cells and can be mediated through the minimum -227 to +77 promoter of the gene encoding PKC $\alpha$ . SOX9 overexpression did not significantly influence PKC $\alpha$ -promoter-dependent luciferase activity in SW480 human CRC cells, but this might be due to the high endogenous SOX9 in these cells, compared with HT29C116E and HCT116 cells (Fig. 1D). Inversely, the activities of the -1571 to +227 and -227 to +77 PKC $\alpha$ -promoter-luciferase constructs were increased from 2 to 4.5-fold in both HT29C116E and HCT116 cells, and up to 8.5 fold in SW480 cells, in response to antisense SOX9 (Blache et al., 2004; Darido et al., 2008), suggesting that PKC $\alpha$  expression is repressed by endogenous SOX9 in these cells. Consistent with this, high SOX9 levels correlated inversely with low levels of mRNA encoding PKC $\alpha$  and of PKC $\alpha$  protein in these CRC cell lines (Fig. 1D-F).

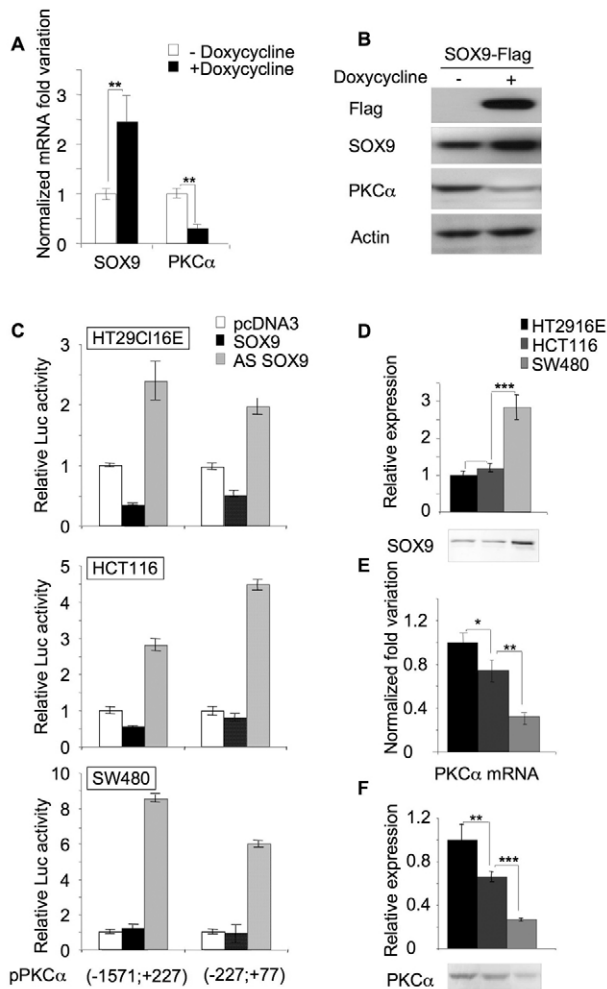
### SOX9 is able to repress PKC $\alpha$ expression in vivo

In normal small intestine, SOX9 is highly expressed and concentrates in cell nuclei of the proliferative crypt compartment, and gradually disappears along the crypt-villus axis during differentiation (Fig. 2A). Inversely, the amount of PKC $\alpha$  is very low within the crypt and progressively increases during differentiation. Similar patterns were observed in the colon epithelium and in HT29C116E cells, which exhibit the ability to differentiate into the goblet-cell lineage when maintained as confluent cultures. This spontaneous differentiation is associated with a decrease in SOX9 expression (Jay et al., 2005) and correlates with the disappearance of SOX9 from the nucleus after 23 days in culture (Fig. 2B), and the concomitant increase in PKC $\alpha$  expression (Fig. 2C).

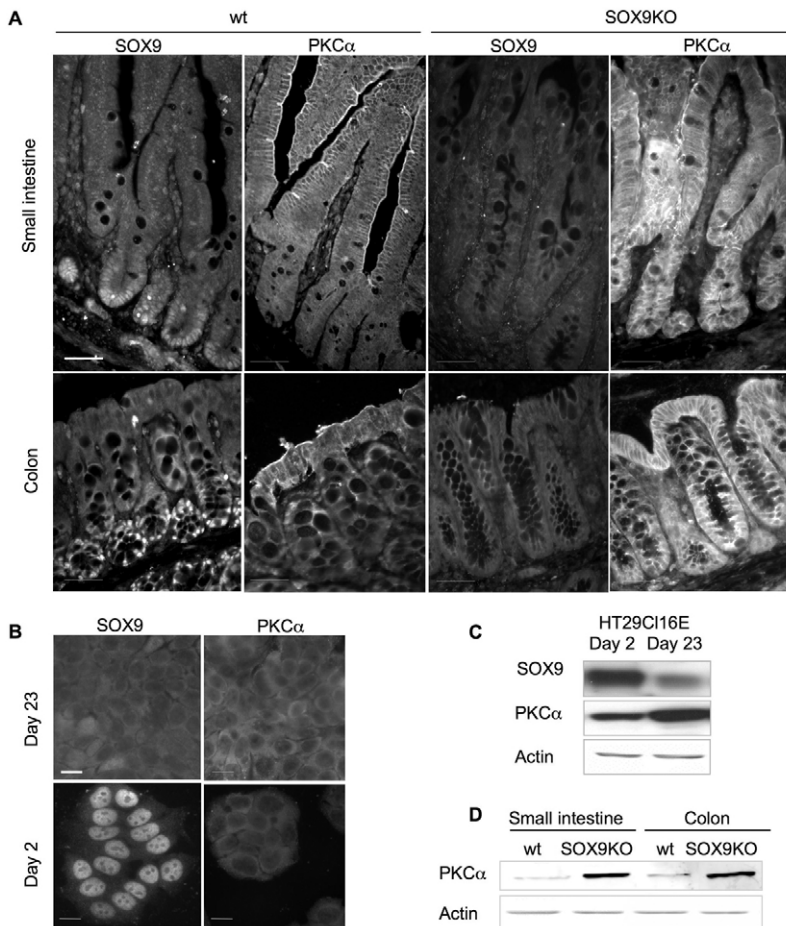
There was clear evidence of SOX9-dependent PKC $\alpha$  repression by the marked increase of PKC $\alpha$  immunostaining from the bottom to the top of the crypt region in both the small intestine and colon from mice that were deficient for SOX9 in the intestinal epithelium (Bastide et al., 2007) (Fig. 2A). The marked PKC $\alpha$  staining of villus cells is intriguing, but might result from the persistence of PKC $\alpha$  in SOX9-deficient crypt cells up to the time of their differentiation. Previous data reported a long half-life for PKC $\alpha$ , from 6 to more than 24 hours (Borner et al., 1988), whereas renewal of intestinal epithelium required 3-5 days. Finally, the western blot presented in Fig. 2D clearly demonstrates the accumulation of PKC $\alpha$  in small-intestinal and colonic epithelium of SOX9 knockout mice, indicating the loss of SOX9-dependent negative regulation of PKC $\alpha$  expression. PKC $\alpha$  is activated along the crypt-to-villus axis of SOX9-deficient intestinal epithelium (as evidenced by the accumulated enzyme at the cell-cell contacts and brush-border plasma membrane), suggesting that this enzyme might exert a biological function at the bottom of the crypt, where it is normally poorly expressed.

### A new mechanism of SOX9 action

Similar to the other SOX-family members, SOX9 is expected to regulate transcription in association with other transcription factors by bending DNA after association of its HMG domain with the minor groove of the DNA helix at the consensus response sequence [A/T][A/T]CAA[A/T]G (Kamachi et al., 2000). Although no obvious SOX-consensus-binding site was identified on the PKC $\alpha$  (-227 to +77) promoter, SOX9 interaction with the promoter of the



**Fig. 1.** SOX9 is able to repress PKC $\alpha$  transcription in CRC cells. (A,B) Effect of doxycycline-induced FLAG-SOX9 expression on the amounts of (A) PKC $\alpha$  mRNA and (B) PKC $\alpha$  protein in HT29C116E cells. (C) Effect of transiently transfected FLAG-SOX9 and SOX9 antisense (AS) on pPKC $\alpha$  (-1571 to +227)- and pPKC $\alpha$  (-227 to +77)-dependent luciferase activities in HT29C116E, HCT116 and SW480 cells. (D-F) Comparative analysis of SOX9 (D), PKC $\alpha$  protein (E) and PKC $\alpha$  mRNA (F) levels in HT29C116E, HCT116 and SW480 cells. Student's *t*-test: \**P*<0.05; \*\**P*<0.01; \*\*\**P*<0.001.



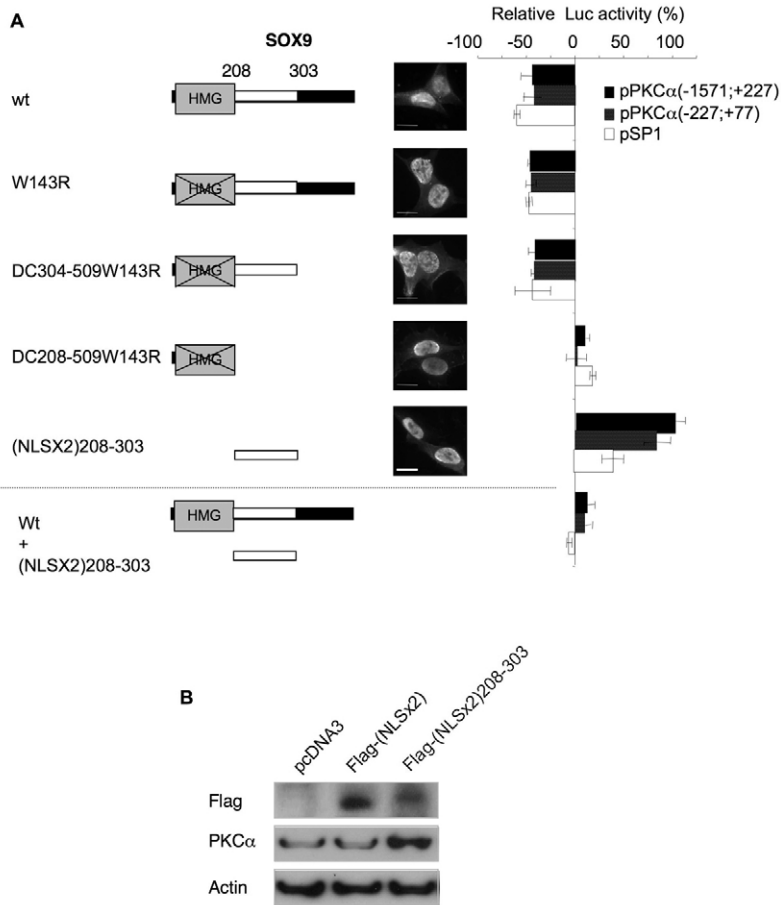
**Fig. 2.** SOX9-dependent PKC $\alpha$  transcriptional repression in vivo. (A,B) SOX9 and PKC $\alpha$  immunostainings in (A) the small intestine and colon of wild-type (wt) mice (left panels) compared with SOX9-deficient (SOX9KO) tissues (right panels) and in (B) proliferating cells (day 2) compared with 'pseudo-differentiated' HT29C116E cells (day 23). (C) Western blot analysis of SOX9 and PKC $\alpha$  levels in total extracts from proliferating and 'pseudo-differentiated' HT29C116E cells. (D) Western blot analysis of PKC $\alpha$  levels in the small intestine and colon epithelium of wild-type mice compared with SOX9-knockout mice. Scale bars: 120  $\mu$ m (A); 15  $\mu$ m (B).

gene encoding PKC $\alpha$  was not necessarily excluded owing to the degeneracy of the SOX-binding site and recent findings about a SOX9-dependent transcription of S100A1 and S100B through a SOX9-binding site distinct from the consensus sequence (Saito et al., 2007). SOX9-dependent chromatin immunoprecipitation, however, was unable to co-precipitate the PKC $\alpha$  (–227 to +77) minimum promoter (data not shown), suggesting no physical interaction of SOX9 with the PKC $\alpha$  promoter. We therefore suspected an indirect SOX9-dependent transcriptional repression as suggested for CDX2, CEA and claudin 7 (Blache et al., 2004; Darido et al., 2008; Jay et al., 2005). This hypothesis did, however, still imply DNA-binding of the SOX9 HMG domain. As shown in Fig. 3A, the campomelic-dysplasia-associated SOX9 W143R mutant, although unable to bind DNA (Meyer et al., 1997), was located in the nucleus of HT29C116E cells and was still able to repress both the PKC $\alpha$  –1571 to +227 and –227 to +77 promoters in a luciferase assay, suggesting that direct DNA binding of the SOX9 HMG domain is not required for this repression (Fig. 3A, compare wt and W143R SOX9 constructs). Deleting the C-terminal transactivation domain (DC304-509W143R) did not have any significant effect on SOX9-mediated PKC $\alpha$  repression, whereas a SOX9 C-terminal deletion including the +208 to +303 (208-303) central region (DC208-509 W143R) completely abolished the repression of the PKC $\alpha$  –1571 to +227 and –227 to +77 promoters. Moreover, expression of a construct in which the 208-303 SOX9 region was fused to two SV40 T-antigen nuclear localization signals (NLSs) in tandem for appropriate targeting to the nucleus

[SOX9(NLSx2)-208-303] upregulated PKC $\alpha$  –1571 to +227 and –227 to +77 promoter activity and exhibited a dominant-negative effect on SOX9-dependent PKC $\alpha$  repression. Fig. 3B further demonstrates that overexpression of SOX9(NLSx2)-208-303 correlates with increased endogenous PKC $\alpha$  expression. Together, these data indicate that the SOX9 (208-303) region is a crucial element in SOX9-dependent repression of transcription of the gene encoding PKC $\alpha$ . This region is highly conserved among the SOXE members (SOX8, SOX9 and SOX10). In SOX8, it exhibits a transactivation potential (Schepers et al., 2000) and, in SOX10, it is involved in the development of glial-cell lineages (Schreiner et al., 2007). Therefore, another biological function can be attributed to the SOX9 (208-303) region, i.e. its involvement in a new DNA-binding-independent transcriptional repression mechanism.

#### SOX9-dependent PKC $\alpha$ repression involves SP1

The DC304-509W143R SOX9 construct, which lacks the transactivation domain, is still able to repress PKC $\alpha$  transcription. Such a construct has previously been useful to show SOX9-dependent inhibition of RUNX2 transcriptional activity through the direct interaction of the SOX9 HMG domain and RUNX2 Runt domain (Zhou et al., 2006). We identified a putative binding site for the AML1 (also known as RUNX1) transcription factor in position –215 to –210 of the promoter of the gene encoding PKC $\alpha$  (Fig. 4A). However, neither a siRNA against *AML1* (decreasing the level of *AML1* mRNA by up to 60%) nor a mutation of the putative AML1-binding site had any effect on the level of mRNA

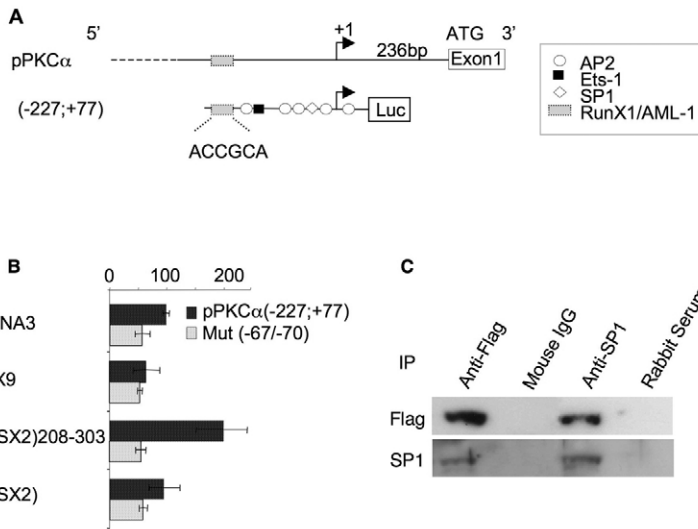


**Fig. 3.** PKC $\alpha$  repression does not require binding of the SOX9 HMG domain to DNA but involves the central domain (208-303). (A) Activity of wild-type (wt), truncated and mutated FLAG-SOX9 on pPKC $\alpha$  (-1571 to +227)-, pPKC $\alpha$  (-227 to +77)- and pSP1-dependent luciferase activities in HT29C116E cells: all FLAG-SOX9 constructs retain nuclear localization, as shown by immunocytochemistry using an anti-FLAG antibody. The C-terminal deletion including the SOX9 (208-303) central region disrupts SOX9-induced transcriptional repression and SOX9 (208-303) fused with two SV40 T-antigen NLSs is able to revert SOX9-dependent transcriptional repression of the activity of all promoters. (B) Western blot analysis indicating the effect of SOX9(NLSx2)208-303 expression on endogenous PKC $\alpha$  expression. Scale bar: 15  $\mu$ m.

et al., 2006). In order to determine whether SOX9 might exert transcriptional repression through SP1, we first investigated the effect of SOX9 on an artificial promoter containing only SP1-binding sites (Sowa et al., 1997). As shown in Fig. 3A, overexpression of wild-type or mutated SOX9 influenced the activity of the pSP1 promoter in a similar manner as observed for the PKC $\alpha$  promoter in HT29C116E cells. Moreover, a mutation disrupting the SP1-binding site drastically decreased the activity of the minimum PKC $\alpha$  promoter and abolished its sensitivity to the wild-type and SOX9(NLSx2)208-303 constructs (Fig. 4B). Finally, Fig. 4C demonstrates that SOX9 co-precipitates with SP1 and is thus able to physically interact with SP1 in our cellular model. Wissmuller et al. reported that the SOX8 or SOX10 HMG domain alone was sufficient to co-precipitate SP1 in their GST-pulldown assays, and that the interaction involved a short conserved sequence located within the SOXE HMG-domain C-terminus (Wissmuller et al., 2006). This sequence apparently is able to bind a number of transcription factors and might be involved in the SOX9-SP1 interaction. Our data further indicate that, in HT29C116E cells, the SOX9 (208-303) central region is required for SOX9-mediated PKC $\alpha$  repression, possibly by increasing the affinity and/or stabilizing the interaction between SOX9 and SP1 within a transcriptional repressor complex.

In summary, the present study indicates that PKC $\alpha$  is a new SOX9 target gene in intestinal epithelium and reveals a new DNA-binding-

encoding PKC $\alpha$  or on PKC $\alpha$  (-227 to +77)-dependent luciferase activity, indicating that SOX9-dependent PKC $\alpha$  repression is not mediated through a similar AML1-dependent mechanism (data not shown). The PKC $\alpha$  promoter, however, does exhibit a functional SP1-binding site (Clark et al., 2002) (Fig. 4A), and previous GST-pulldown data from Wissmuller et al. indicate that SP1 is able to interact with the SOXE members SOX8 and SOX10 (Wissmuller



**Fig. 4.** Involvement of SP1 in the SOX9-dependant transcriptional repression of the gene encoding PKC $\alpha$ . (A) Schematic representation of the -227 to +77 PKC $\alpha$  promoter fused to the luciferase-reporter gene, indicating the previously identified transcription start site, the binding sites of the transcription factors AP2, ets-1 and SP1 (Clark et al., 2002), and a potential AML1-binding site in position -215 to -210. (B) Luciferase assay comparing the properties of the wild-type PKC $\alpha$  promoter and the -227 to +77 PKC $\alpha$  promoter mutated within the SP1-binding site [mut (-67/-70)] (Clark et al., 2002). Note the insensitivity of the mutated promoter in response to wild-type and SOX9(NLSx2)-208-303. (C) Western blot demonstrating co-precipitation of SOX9 and SP1 from FLAG-SOX9-induced HT29C116E whole-cell extracts.

independent mechanism for SOX9. Because SOX9 is upregulated by the Wnt-APC pathway in intestinal epithelial cells (Blache et al., 2004), our results provide an explanation for the correlation that is observed in both normal and malignant intestinal epithelium between activated Wnt-APC signaling, upregulation of SOX9 (Cardoso et al., 2006) and reduced expression of PKC $\alpha$  (Oster and Leitges, 2006). PKC $\alpha$  overexpression has been associated with cell-growth arrest and differentiation (Scaglione-Sewell et al., 1998), and PKC $\alpha$  knockout has been associated with tumor formation in the intestine epithelium (Oster and Leitges, 2006). However, in SOX9-deficient intestine overexpressing PKC $\alpha$ , neither reduced proliferation nor increased differentiation was observed; instead, hyperproliferation, no paneth cells and fewer goblet cells were found (Bastide et al., 2007). This result then raises the issue of the functional role of SOX9-dependent PKC $\alpha$  repression in colorectal cancer. Besides PKC $\alpha$  repression, SOX9 also exerts a negative-feedback loop on the Wnt-APC pathway (Bastide et al., 2007) and, therefore, it is possible that cell-growth inhibition due to PKC $\alpha$  overexpression is not sufficient to overcome the increase in proliferation caused by upregulation of the Wnt-APC pathway in intestine that is deficient for SOX9. Previous data from our laboratory indicate that SOX9-dependent inhibition of the Wnt-APC pathway involves the classic mechanism of SOX9 action, i.e. the binding of SOX9 to DNA (Bastide et al., 2007). This inhibition might result from an upregulation of groucho-related inhibitors of the  $\beta$ -catenin–Tcf complex (Bastide et al., 2007) and of CEACAM1, a direct SOX9 target (Jin et al., 2008; Leung et al., 2008; Zalzal et al., 2008). Indeed, the W143R SOX9 mutant, which is unable to bind DNA, is unable to inhibit the Wnt-APC pathway, although it is able to repress PKC $\alpha$  expression, as shown in the present study. Because the DNA-binding-dependent activity of endogenous SOX9 is weak in colon cancer cells (Darido et al., 2008), it may be postulated that the SOX9-dependent negative-feedback loop on the Wnt-APC pathway is at least partially lost in CRC cells. By contrast, PKC $\alpha$  repression, which does not require SOX9 DNA-binding, is maintained, thus favoring proliferation, inhibiting differentiation and potentially facilitating tumor progression, particularly in the context of a constitutively activated Wnt-APC pathway. Elucidating the causes of the decrease of SOX9 DNA-binding-dependent activity in CRC cells is now an attractive issue to address, and might provide a new approach to influence the balance between proliferation and differentiation of CRC cells.

## Materials and Methods

### Cell cultures

HT29C116E, HCT116, SW480 and the SOX9-inducible HT29C116E cells were cultured and induced as previously described (Jay et al., 2005).

### Plasmids

The reporter constructs containing the human pPKC $\alpha$  and the pSP1 promoters were previously described (Clark et al., 2002). The putative AML1-binding site in position –215 to –210 was disrupted using the oligonucleotide 5'-GTGTTCCAGC-ATTGTCAGGCACTCGCTGCCTCCTCC-3' and its complementary sequence, as was performed for the murine *Ada* gene (Schaubach et al., 2006). The FLAG-tagged wild-type and truncated versions of SOX9 exhibiting the W143R mutation are described elsewhere (Jay et al., 2005; Bastide et al., 2007). The SOX9(NLSx2)208-303 construct was obtained with a synthetic insert encoding the FLAG and two SV40 T-antigen NLSs (5'-ATGGACTACAAGGACGACGATGACAAGGGTACCGAT-CCAAAAAAGAAGAGAAAGGTAGATCCAAAAAAGAAGAGAAAGGTA-3') subcloned in frame with the SOX9 (208-303) region within the pcDNA3 polylinker. The SOX9 antisense construct has been previously described (Blache et al., 2004).

### Luciferase assays

Assays were performed in triplicate, at least three independent times as in Blache et al. (Blache et al., 2004). Transfection efficiencies were normalized by co-transfecting

the pHRG-TK standardization vector (Promega). Results are expressed as the ratio of luciferase activity relative to the control pGL3 and pcDNA3 empty plasmids.

### Western blotting

Equal amounts of total-protein extracts from cultured cells (30  $\mu$ g) or mice intestinal epithelium (20  $\mu$ g) obtained by scraping the inner intestine layer were loaded on a 10% SDS-PAGE and transferred onto PVDF membranes. Primary antibodies were the mouse anti-PKC $\alpha$  (1:1000; Upstate Biotechnology), mouse anti-FLAG (1:500; Sigma), rabbit anti-SOX9 (1:1000) (Bastide et al., 2007), mouse anti-actin (1:5000; Sigma), rabbit anti-SP1 (1:200; Santa Cruz Biotechnology). Secondary antibodies were the IRdyeTM 800- and IRdyeTM 700DX-conjugated anti-mouse and rabbit (1:10,000; Tebu-Bio). Results were analyzed using the OdysseyR infrared imaging system (LI-COR Biosciences).

### Immunoprecipitation

Immunoprecipitations were performed with magnetic beads (Ademtech), 1 mg SOX9-induced HT29C116E whole-cell extracts and 2  $\mu$ g of either the anti-FLAG or the anti-SP1 antibodies, according to Ademtech recommendations with a mouse anti-IgG (Santa Cruz Biotechnology) or a rabbit serum as controls.

### Real-time RT-PCR

Experiments were performed with the following primer pairs: GAPDH, 5'-GAC-CACAGTCCATGCCATCACT-3' and 5'-TCCACCACCTGTGCTGTAG-3'; SOX9, 5'-GCCAGGTGCTCAAAGGCTA-3' and 5'-TCTCGTTCAAGAAG-TCTCCAGAG-3'; PKC $\alpha$ , 5'-GCTTCCAGTGCCAAGTTTGC-3' and 5'-GCACC-CGGACAAGAAAAAGTAA-3' (Bastide et al., 2007). Results are expressed as the variation of the studied transcript standardized to GAPDH and relative to the control conditions.

### Immunofluorescence

Paraffin-embedded intestine sections were obtained as previously described (Bastide et al., 2007). HT29C116E cells were plated in 24-well plates (50,000/well) on coverslips. Antibodies were the rabbit anti-SOX9 (1:200), rabbit anti-PKC $\alpha$  (1:400; Sigma) and the Cy3-conjugated secondary anti-rabbit antibody (1:1000; Santa Cruz Biotechnology). Preparations were mounted in Mowiol and observed using an epifluorescent microscope (Zeiss).

### siRNA

HT29C116E cells (350,000) were seeded in 60-mm dishes and transfected with 100 nM of an siRNA targeting the *AML1* messenger sequence 5'-CCGCCGUU-CACGCCGCCUUC-3' (Wang et al., 2005). After 48 hours, cells were harvested and mRNA content was analyzed by real-time RT-PCR.

### Chromatin immunoprecipitation

Chromatin immunoprecipitation (ChIP) assays were performed using the Magna ChIP G Kit and a ChIPable HT29 chromatin (Upstate) with either the rabbit anti-SOX9 or rabbit serum as a control. Forward 5'-GTGTTCCAGCACCAGCAAGG-3' and reverse 5'-GAGAGTCGGGCTGGTGCTG-3' primers were used to amplify the PKC $\alpha$  –227 to +77 minimum promoter by PCR.

This work was supported by Institut National de la Santé et de la Recherche Médicale (INSERM), Centre national de la Recherche Scientifique (CNRS), Agence Nationale pour la Recherche (ANR), Association pour la Recherche contre le Cancer (ARC) (No. 3570 and 3636), Ligue Nationale contre le Cancer (Equipe Labellisée), Fondation pour la Recherche Médicale, Groupement des Entreprises Françaises dans la Lutte contre le Cancer (GEFLUC), Ligue Régionale contre le Cancer, CNRS du LIBAN and Ministère de l'Enseignement Supérieur et de La Recherche.

## References

- Bastide, P., Darido, C., Pannequin, J., Kist, R., Robine, S., Marty-Double, C., Bibeau, F., Scherer, G., Joubert, D., Hollande, F. et al. (2007). Sox9 regulates cell proliferation and is required for Paneth cell differentiation in the intestinal epithelium. *J. Cell Biol.* **178**, 635–648.
- Blache, P., van de Wetering, M., Duluc, I., Domon, C., Berta, P., Freund, J. N., Clevers, H. and Jay, P. (2004). SOX9 is an intestine crypt transcription factor, is regulated by the Wnt pathway, and represses the CDX2 and MUC2 genes. *J. Cell Biol.* **166**, 37–47.
- Borner, C., Eppenberger, U., Wyss, R. and Fabbro, D. (1988). Continuous synthesis of two protein-kinase-C-related proteins after down-regulation by phorbol esters. *Proc. Natl. Acad. Sci. USA* **85**, 2110–2114.
- Cardoso, J., Molenaar, L., de Menezes, R. X., van Leerdam, M., Rosenberg, C., Moslein, G., Sampson, J., Morreau, H., Boer, J. M. and Fodde, R. (2006). Chromosomal instability in MYH- and APC-mutant adenomatous polyps. *Cancer Res.* **66**, 2514–2519.
- Clark, J. H., Haridas, V. and Glazer, R. I. (2002). Modulation of the human protein kinase C alpha gene promoter by activator protein-2. *Biochemistry* **41**, 11847–11856.

- Darido, C., Buchert, M., Pannequin, J., Bastide, P., Zalali, H., Mantamadiotis, T., Bourgaux, J. F., Garambois, V., Jay, P., Blache, P. et al. (2008). Defective claudin-7 regulation by Tcf-4 and Sox-9 disrupts the polarity and increases the tumorigenicity of colorectal cancer cells. *Cancer Res.* **68**, 4258-4268.
- De Santa Barbara, P., Bonneaud, N., Boizet, B., Desclozeaux, M., Moniot, B., Sudbeck, P., Scherer, G., Poulat, F. and Berta, P. (1998). Direct interaction of SRY-related protein SOX9 and steroidogenic factor 1 regulates transcription of the human anti-Mullerian hormone gene. *Mol. Cell. Biol.* **18**, 6653-6665.
- Episkopou, V. (2005). SOX2 functions in adult neural stem cells. *Trends Neurosci.* **28**, 219-221.
- Gokmen-Polar, Y., Murray, N. R., Velasco, M. A., Gatalica, Z. and Fields, A. P. (2001). Elevated protein kinase C beta11 is an early promotive event in colon carcinogenesis. *Cancer Res.* **61**, 1375-1381.
- Jay, P., Berta, P. and Blache, P. (2005). Expression of the carcinoembryonic antigen gene is inhibited by SOX9 in human colon carcinoma cells. *Cancer Res.* **65**, 2193-2198.
- Jin, L., Li, Y., Chen, C. J., Sherman, M. A., Le, K. and Shively, J. E. (2008). Direct interaction of tumor suppressor CEACAM1 with beta catenin: identification of key residues in the long cytoplasmic domain. *Exp. Biol. Med. (Maywood)* **233**, 849-859.
- Kahl-Rainer, P., Karner-Hanusch, J., Weiss, W. and Marian, B. (1994). Five of six protein kinase C isoenzymes present in normal mucosa show reduced protein levels during tumor development in the human colon. *Carcinogenesis* **15**, 779-782.
- Kamachi, Y., Uchikawa, M. and Kondoh, H. (2000). Pairing SOX off: with partners in the regulation of embryonic development. *Trends Genet.* **16**, 182-187.
- Kanai, Y., Hiramatsu, R., Matoba, S. and Kidokoro, T. (2005). From SRY to SOX9: mammalian testis differentiation. *J. Biochem.* **138**, 13-19.
- Lee, Y. H., Aoki, Y., Hong, C. S., Saint-Germain, N., Credidio, C. and Saint-Jeannet, J. P. (2004). Early requirement of the transcriptional activator Sox9 for neural crest specification in *Xenopus*. *Dev. Biol.* **275**, 93-103.
- Leontieva, O. V. and Black, J. D. (2004). Identification of two distinct pathways of protein kinase C alpha down-regulation in intestinal epithelial cells. *J. Biol. Chem.* **279**, 5788-5801.
- Leung, N., Turbide, C., Balachandra, B., Marcus, V. and Beauchemin, N. (2008). Intestinal tumor progression is promoted by decreased apoptosis and dysregulated Wnt signaling in *Ceacam1<sup>-/-</sup>* mice. *Oncogene* **27**, 4943-4953.
- Meyer, J., Sudbeck, P., Held, M., Wagner, T., Schmitz, M. L., Bricarelli, F. D., Eggermont, E., Friedrich, U., Haas, O. A., Kobelt, A. et al. (1997). Mutational analysis of the SOX9 gene in campomelic dysplasia and autosomal sex reversal: lack of genotype/phenotype correlations. *Hum. Mol. Genet.* **6**, 91-98.
- Murray, N. R., Davidson, L. A., Chapkin, R. S., Clay Gustafson, W., Schattenberg, D. G. and Fields, A. P. (1999). Overexpression of protein kinase C beta11 induces colonic hyperproliferation and increased sensitivity to colon carcinogenesis. *J. Cell Biol.* **145**, 699-711.
- Ng, L. J., Wheatley, S., Muscat, G. E., Conway-Campbell, J., Bowles, J., Wright, E., Bell, D. M., Tam, P. P., Cheah, K. S. and Koopman, P. (1997). SOX9 binds DNA, activates transcription, and coexpresses with type II collagen during chondrogenesis in the mouse. *Dev. Biol.* **183**, 108-121.
- Oster, H. and Leitges, M. (2006). Protein kinase C alpha but not PKCzeta suppresses intestinal tumor formation in *ApcMin/+* mice. *Cancer Res.* **66**, 6955-6963.
- Perletti, G. P., Folini, M., Lin, H. C., Mischak, H., Piccinini, F. and Tashjian, A. H., Jr (1996). Overexpression of protein kinase C epsilon is oncogenic in rat colonic epithelial cells. *Oncogene* **12**, 847-854.
- Saito, T., Ikeda, T., Nakamura, K., Chung, U. I. and Kawaguchi, H. (2007). S100A1 and S100B, transcriptional targets of SOX trio, inhibit terminal differentiation of chondrocytes. *EMBO Rep.* **8**, 504-509.
- Saxon, M. L., Zhao, X. and Black, J. D. (1994). Activation of protein kinase C isozymes is associated with post-mitotic events in intestinal epithelial cells in situ. *J. Cell Biol.* **126**, 747-763.
- Scaglione-Sewell, B., Abraham, C., Bissonnette, M., Skarosi, S. F., Hart, J., Davidson, N. O., Wali, R. K., Davis, B. H., Sitrin, M. and Brasitus, T. A. (1998). Decreased PKC-alpha expression increases cellular proliferation, decreases differentiation, and enhances the transformed phenotype of CaCo-2 cells. *Cancer Res.* **58**, 1074-1081.
- Schaubach, B. M., Wen, H. Y. and Kellems, R. E. (2006). Regulation of murine Ada gene expression in the placenta by transcription factor RUNX1. *Placenta* **27**, 269-277.
- Scheppers, G. E., Bullejos, M., Hosking, B. M. and Koopman, P. (2000). Cloning and characterisation of the Sry-related transcription factor gene Sox8. *Nucleic Acids Res.* **28**, 1473-1480.
- Schreiner, S., Cossais, F., Fischer, K., Scholz, S., Bosl, M. R., Holtmann, B., Sendtner, M. and Wegner, M. (2007). Hypomorphic Sox10 alleles reveal novel protein functions and unravel developmental differences in glial lineages. *Development* **134**, 3271-3281.
- Shimizu, H., Yokoyama, S. and Asahara, H. (2007). Growth and differentiation of the developing limb bud from the perspective of chondrogenesis. *Dev. Growth Differ.* **49**, 449-454.
- Sowa, Y., Orita, T., Minamikawa, S., Nakano, K., Mizuno, T., Nomura, H. and Sakai, T. (1997). Histone deacetylase inhibitor activates the WAF1/Cip1 gene promoter through the Sp1 sites. *Biochem. Biophys. Res. Commun.* **241**, 142-150.
- Suga, K., Sugimoto, I., Ito, H. and Hashimoto, E. (1998). Down-regulation of protein kinase C-alpha detected in human colorectal cancer. *Biochem. Mol. Biol. Int.* **44**, 523-528.
- Wang, Y., Belflower, R. M., Dong, Y. F., Schwarz, E. M., O'Keefe, R. J. and Drissi, H. (2005). Runx1/AML1/Cbfa2 mediates onset of mesenchymal cell differentiation toward chondrogenesis. *J. Bone Miner. Res.* **20**, 1624-1636.
- Wissmuller, S., Kosian, T., Wolf, M., Finzsch, M. and Wegner, M. (2006). The high-mobility-group domain of Sox proteins interacts with DNA-binding domains of many transcription factors. *Nucleic Acids Res.* **34**, 1735-1744.
- Zalali, H., Naudin, C., Bastide, P., Quittau-Prevostel, C., Yaghi, C., Poulat, F., Jay, P. and Blache, P. (2008). CEACAM1, a SOX9 direct transcriptional target identified in the colon epithelium. *Oncogene* **27**, 7131-7138.
- Zhou, G., Zheng, Q., Engin, F., Munivez, E., Chen, Y., Sebald, E., Krakow, D. and Lee, B. (2006). Dominance of SOX9 function over RUNX2 during skeletogenesis. *Proc. Natl. Acad. Sci. USA* **103**, 19004-19009.
- Zorn, A. M. and Wells, J. M. (2007). Molecular basis of vertebrate endoderm development. *Int. Rev. Cytol.* **259**, 49-111.