

# Calcium Sensing Receptor in Human Colon Carcinoma: Interaction with $\text{Ca}^{2+}$ and 1,25-Dihydroxyvitamin $\text{D}_3$

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## Abstract

Recent studies show that the human parathyroid calcium sensing receptor (CaSR) is expressed in human colon epithelium and functions to regulate epithelial proliferation and differentiation. In this study, we show that the cells of the colon crypt acquire CaSR expression as they differentiate and migrate towards the apex of the crypt. CaSR expression was weak in colon carcinomas with a more-differentiated histologic pattern, whereas CaSR expression was undetectable in less-differentiated tumors. We found that  $\text{Ca}^{2+}$  and/or 1,25(OH) $_2\text{D}_3$  stimulated CaSR promoter activity and CaSR protein expression in the human colon carcinoma CBS cells, which possessed a functional CaSR. Both agents concomitantly induced a series of changes in the CBS cells that influence proliferation and differentiation, but cellular responses to the two agents were not identical.  $\text{Ca}^{2+}$  strongly induced E-cadherin expression and inhibited the expression of the nuclear transcription factor, TCF4. 1,25(OH) $_2\text{D}_3$  was weaker in its effect on E-cadherin and was not able to inhibit TCF4 expression. 1,25(OH) $_2\text{D}_3$  was as strong or stronger than  $\text{Ca}^{2+}$  in its induction of the cyclin-dependent kinase inhibitors, P21 and p27. It is concluded that CaSR may function in the colon to regulate epithelial differentiation and that loss of CaSR expression may be associated with abnormal differentiation and/or malignant progression. Extracellular  $\text{Ca}^{2+}$  and 1,25(OH) $_2\text{D}_3$  are potential candidates involved in regulating CaSR expression in the colon and the chemopreventive actions of  $\text{Ca}^{2+}$  and 1,25(OH) $_2\text{D}_3$  in colon cancer may be mediated, in part, through the CaSR. (Cancer Res 2005; 65(2): 493-8)

## Introduction

The human parathyroid calcium sensing receptor (CaSR) senses minute changes in extracellular  $\text{Ca}^{2+}$  concentration and functions to tightly regulate systemic  $\text{Ca}^{2+}$  homeostasis (1). There is increasing evidence to suggest that the function of the CaSR is diverse, extends beyond the systemic control of  $\text{Ca}^{2+}$  homeostasis, and is contingent on the tissue type in which the receptor is expressed (2–5).  $\text{Ca}^{2+}$  is a chemopreventive agent for colon cancer (6, 7). How it acts at the molecular level to inhibit or delay carcinogenesis in the colon, however, is not understood. We and others have recently found that the CaSR is expressed in human colon epithelium (8, 9). Compared with normal colon crypt epithelial cells, a loss of CaSR expression is observed in

differentiated carcinomas, whereas little or no CaSR expression is found in undifferentiated and invasive carcinomas (9). Thus, the expression of CaSR may be associated with abnormal differentiation or malignant progression, or both.

E-Cadherin is an epithelial homophilic transmembrane glycoprotein and functions to mediate epithelial cell-cell adhesion and maintain the integrity of the epithelium (10–12). Loss of E-cadherin expression is associated with many forms of epithelial cancer (13–17) and ectopic expression of functional E-cadherin in transformed cells results in the suppression of their transformed phenotype (18, 19). Thus, E-cadherin functions as a tumor suppressor. Exactly how E-cadherin acts to suppress the transformed phenotype at the molecular level, however, is not fully understood. E-cadherin interacts with  $\beta$ -catenin, a component of the Wnt family of signaling pathways (10, 20–22). Activation of Wnt allows the accumulation of  $\beta$ -catenin in the nucleus. In the nucleus,  $\beta$ -catenin interacts with and activates the lymphoid enhancer factor-T cell factor family of transcription factors (TCF) and drives the expression of a variety of malignant genes and promotes the malignant phenotype (23).

Human colon carcinoma cell lines such as CBS and Moser, derived from differentiated human primary colon tumors, possess a functional CaSR (9). Because the cell-impermeable CaSR agonist  $\text{Gd}^{3+}$  (2–4) mimics the molecular effect of extracellular  $\text{Ca}^{2+}$ , this suggests that CaSR expression may be directly linked to the mechanism by which extracellular  $\text{Ca}^{2+}$  suppresses the malignant phenotype in colon carcinoma cells (9). Therefore, these cells offer an opportunity to investigate how the CaSR functions to control the biological phenotype. Our previous studies show that the CaSR functions to promote differentiation (or suppress malignant properties) in these colon carcinoma cells by up-regulating the expression of E-cadherin and down-regulating the binding of  $\beta$ -catenin to TCF4 (9). Interestingly, the active metabolite of vitamin D, 1,25(OH) $_2\text{D}_3$ , another chemopreventive agent for colon cancer, also acts through the E-cadherin and  $\beta$ -catenin/TCF pathways (24). An important physiologic function of vitamin D is to increase  $\text{Ca}^{2+}$  absorption in the gut (25). The *CaSR* gene possesses two promoters, each containing a vitamin D response element (VDRE; ref. 26). Thus, a mechanistic relationship is likely to exist between the action of 1,25(OH) $_2\text{D}_3$  and that of  $\text{Ca}^{2+}$  at both the physiologic and molecular levels.

Because loss of CaSR expression seems to be associated with abnormal differentiation (9), we investigated the expression of the CaSR in normal human colon crypt epithelial cells and focused on CaSR expression in different compartments of the colon crypt. In this study, we showed that the rapidly proliferating crypt epithelial cells at the bottom of the crypts did not express the CaSR. The CaSR was expressed in cells in the middle of the crypt and its expression was most intense in cells at the top of the crypt. Because the

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expression of the CaSR increases as the cells migrate up the crypt, we hypothesized that  $\text{Ca}^{2+}$  and  $1,25(\text{OH})_2\text{D}_3$  function to stimulate CaSR expression. This hypothesis was tested in the human colon carcinoma CBS cells. We found that both promoters of the *CaSR* gene were active in these cells and were stimulated by  $\text{Ca}^{2+}$  or  $1,25(\text{OH})_2\text{D}_3$ . Combination of  $\text{Ca}^{2+}$  and  $1,25(\text{OH})_2\text{D}_3$  stimulated the promoter 2 (P2) of the *CaSR* gene in an additive manner.  $\text{Ca}^{2+}$  and/or  $1,25(\text{OH})_2\text{D}_3$  stimulated CaSR expression in these cells. Combination of  $\text{Ca}^{2+}$  and  $1,25(\text{OH})_2\text{D}_3$  was also found to enhance the stimulation of E-cadherin expression. The similarity and differences in the molecular action of  $\text{Ca}^{2+}$  and  $1,25(\text{OH})_2\text{D}_3$  were also investigated in this study using molecular markers that are functionally associated with the induction of differentiation in colon carcinomas (19, 27–29). We found that both  $\text{Ca}^{2+}$  and  $1,25(\text{OH})_2\text{D}_3$  stimulated the expression of E-cadherin, the cyclin-dependent kinase inhibitors p21/Waf1 and p27, and  $\gamma$ -catenin. In addition,  $\text{Ca}^{2+}$ , but not  $1,25(\text{OH})_2\text{D}_3$ , suppressed the expression of TCF4.

It is concluded that colon crypt epithelial cells acquire CaSR expression as they migrate and differentiate towards the apex of the crypt. Both  $\text{Ca}^{2+}$  and  $1,25(\text{OH})_2\text{D}_3$  are good candidate molecules in stimulating the expression of CaSR and the two agents act in an overlapping but distinct manner.

## Materials and Methods

**Cell culture, Treatment with  $\text{Ca}^{2+}$  and  $1,25(\text{OH})_2\text{D}_3$ .** CBS colon carcinoma cells were maintained in  $\text{Ca}^{2+}$ -free SMEM medium (Sigma, St. Louis, MO) supplemented with sodium bicarbonate, peptone, vitamins, amino acids, and 5% fetal bovine serum as described previously (9). The medium of actively growing cells was replenished with medium containing 1 mmol/L  $\text{Ca}^{2+}$ , or 0.1  $\mu\text{mol/L}$   $1,25(\text{OH})_2\text{D}_3$ , or 1.0  $\mu\text{mol/L}$   $1,25(\text{OH})_2\text{D}_3$ , or a combination of  $\text{Ca}^{2+}$  and  $1,25(\text{OH})_2\text{D}_3$  for the time periods as indicated in the figure legends.

**Immunoblotting and CaSR Promoter Reporter Assay.** Immunoblotting was done using commercially available polyclonal or monoclonal antibodies as previously described (9, 27). CaSR promoter reporter assays were done using the Promega dual luciferase system (Promega Co., Madison, WI) and CaSR P1 and P2 promoter constructs (26). Details of the constructs used (P1-VDRE WT, P1-VDRE MUT, P2-VDRE WT, and P2-VDRE MUT) can be found in Fig. 8 of ref. (26). Luciferase activities were normalized to transfection efficiency by cotransfection with pRL *Renilla* luciferase construct.

Cells were plated in 6-well culture plates and transfected with 1  $\mu\text{g}$  of CASR P1 or P2 promoter construct and 0.2  $\mu\text{g}$  *Renilla* construct in each well using Fugene 6 transfection reagent. Eight hours later, the cells were exposed to a final concentration of 1 mmol/L  $\text{Ca}^{2+}$  or 0.1 mol/L  $1,25(\text{OH})_2\text{D}_3$ , or a combination of  $\text{Ca}^{2+}$  and  $1,25(\text{OH})_2\text{D}_3$  in medium containing 2.5% fetal bovine serum. Cells were exposed to  $\text{Ca}^{2+}$  or  $1,25(\text{OH})_2\text{D}_3$ , or  $\text{Ca}^{2+}$  and  $1,25(\text{OH})_2\text{D}_3$  for 36 hours, washed in cold PBS, and lysed in lysis buffer. The cell lysates were frozen and thawed twice before measurement of luciferase activities. Luciferase activities of the cell lysates were measured in a Td-20/20 luminometer and normalized to transfection efficiency with *Renilla* luciferase activities.

**Immunohistochemistry.** Immunoperoxidase staining for CaSR was done using a rabbit polyclonal antibody (Affinity BioReagents, Golden, CO) as described previously (9). Five-micrometer sections of formalin-fixed, paraffin-embedded surgical specimens of human colon tumors were stained by the immunoperoxidase method and the reaction product visualized using diaminobenzidine as the chromogenic substrate. Immunostained sections were lightly counterstained with hematoxylin and examined by light microscopy.

The expression of CaSR was evaluated semiquantitatively in the normal epithelial component of nine primary human colon tumor specimens. The normal colon crypt was arbitrarily divided into three zones: basal, mid-

crypt, and surface. The basal zone was defined as the bottom of the crypt where small, densely packed cells without evidence of goblet cell structure were present. The mid-crypt was defined as the area from the upper edge of the basal zone (i.e., beginning where goblet cell structure was evident) to an area 1/3 of the way from the top of the crypt. The surface zone was defined as the upper 1/3 of the crypt and the cells at the apex of the crypt. The percentage of epithelial cells in each zone that were positive for CaSR expression was determined and the staining density of cells was quantitatively scored on a scale of 1 to 4 (1 = barely detectable immunoreactivity and 4 = strongest immunoreactivity). From each specimen, staining was assessed in four separate crypts.

Areas of histologically more differentiated tumor in seven specimens and areas of histologically less differentiated tumor in four of the specimens were examined in the same manner. The percentage of CaSR-positive cells in the tumor tissue as well as staining intensity were determined as with normal tissue.

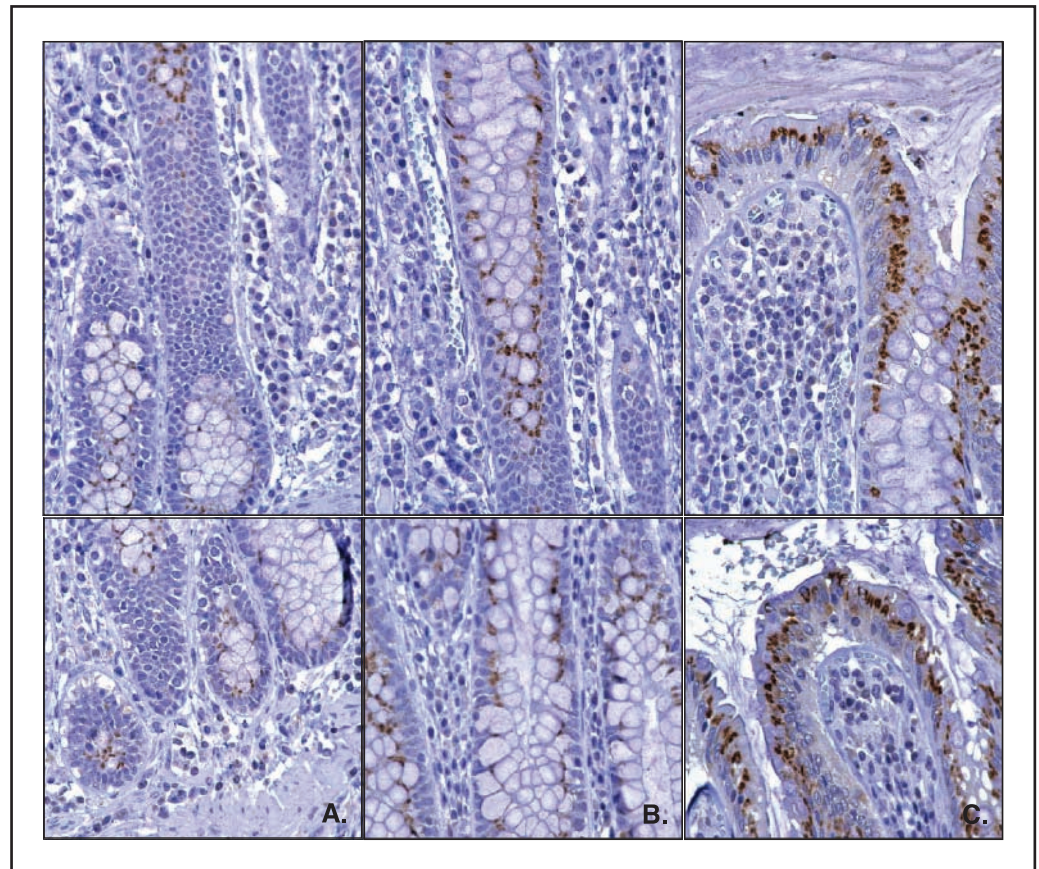
## Results and Discussion

The expression of CaSR was evaluated in the normal component of nine primary human colon carcinoma specimens. There was little CaSR staining of cells in the basal zone (Fig. 1A). An occasional "brown" cell was observed in the basal zone but it was possible that these cells represented cells at the edge of the secretory (mid-crypt) zone. In contrast, virtually all of the cells in mid-crypt (Fig. 1B) and surface zones (Fig. 1C) stained for CaSR. Staining of apical cells was slightly more intense on the average than staining of the mid-crypt cells. Quantitatively, the percentage of cells that were positive for CaSR expression in both the apex and mid-crypt areas and the staining intensity of these cells were significantly different from that seen in the basal zone of the crypt (Table 1).

These results suggest that the CaSR expression is associated with differentiation of the crypt epithelial cells as these cells migrate upwards from the base of the crypt towards the apex. We hypothesized that if the CaSR functions to regulate cellular differentiation, there would be an inverse relationship between CaSR expression and degree of differentiation in colon carcinoma, as well. Previous analyses suggested the existence of such an inverse relationship. In a small sample of tumors, we noted there was relatively little CaSR staining of more-differentiated tumor tissue and virtually no staining in areas of less-differentiated carcinoma (9). In the present study, we have quantitatively assessed CaSR expression in a group of well- to moderately differentiated tumors as well as in four tumors with a poorly differentiated histologic presentation. Tumors were categorized as moderately differentiated to well-differentiated based on evidence of glandular structure, or poorly differentiated based on areas in which only cell sheets were detected (i.e., with no evidence of glandular structure). In addition, small isolated clusters of tumor cells (1-3 cells) imbedded in the stroma were also observed in two specimens. These isolated cells were defined as invasive cells. Representative immunostained sections of a moderately differentiated tumor and a less-differentiated tumor are shown (Fig. 2). In the histologically more differentiated tumor, staining was variable. There were areas in which no staining was detected and areas in which the glandular epithelial cells were virtually 100% positive (Fig. 2A). In the poorly differentiated tumor specimen, the epithelium was almost entirely negative for CaSR reactivity (Fig. 2B). Quantitatively, both the percentage of positive cells and the staining intensity of the carcinoma cells in poorly differentiated tumors were found to be significantly reduced when compared with that of well- to



**Figure 1.** CaSR expression in normal colonic crypt epithelium. A, basal zone; B, mid-crypt; C, surface or apex of the crypt. All sections: ×400.



moderately differentiated tumors (Table 1). Small clusters of isolated epithelial cells in the stroma observed in these two specimens were also nonreactive with the anti-CaSR antibody.

How the expression of the CaSR is regulated at the molecular level is not known. Ca<sup>2+</sup> itself has been proposed as a potential candidate in regulating CaSR expression (26). Interestingly, at the physiologic level, a mechanistic linkage exists between vitamin D and the CaSR because vitamin D functions to increase Ca<sup>2+</sup> absorption in the gut (25). It has been reported that 1,25(OH)<sub>2</sub>D<sub>3</sub> (an active metabolite of vitamin D) functions to promote differentiation of human colon carcinoma cells through the E-cadherin and catenin system (24). Thus, at this level the action of 1,25(OH)<sub>2</sub>D<sub>3</sub> is quite similar to that of Ca<sup>2+</sup> as reported by us (9). At the molecular level, both promoters of the *CaSR* gene possess functional VDREs (26). Based on this, we hypothesized that both Ca<sup>2+</sup> and 1,25(OH)<sub>2</sub>D<sub>3</sub> are involved in regulating CaSR expression. To test this hypothesis, we used the human colon carcinoma CBS cells, which respond to Ca<sup>2+</sup> and the CaSR agonist Gd<sup>3+</sup> (9). The ability of Ca<sup>2+</sup> and 1,25(OH)<sub>2</sub>D<sub>3</sub> to stimulate the P1 and P2 promoters of the *CaSR* gene in CBS cells was assessed using luciferase reporter assays. Both the P1 and P2 promoters of the *CaSR* gene were active in the CBS cells and P2 was relatively more active by comparison with P1 (Fig. 3A). Both extracellular Ca<sup>2+</sup> and 1,25(OH)<sub>2</sub>D<sub>3</sub> stimulated P1 and P2 promoter activities (Fig. 3A). The stimulation of P1 and P2 by 1,25(OH)<sub>2</sub>D<sub>3</sub> in the colon cells was specifically through the VDREs because both P1 and P2 were unresponsive to 1,25(OH)<sub>2</sub>D<sub>3</sub> when the VDREs were mutated (Fig. 3A). On the other hand, mutated forms of both P1 and P2 responded equally well to Ca<sup>2+</sup> as the parental (nonmutated) forms.

**Table 1.** Quantitative assessment of CaSR immunoreactivity in normal colonic crypt epithelium and colon carcinoma.

Histological features	% Positive cells	Staining intensity
Normal colonic crypt epithelium ( <i>n</i> = 9)		
Basal zone	6 ± 1	1.1 ± 0.1
Mid-crypt	90 ± 5*	2.3 ± 1.5 <sup>†</sup>
Surface	96 ± 2*	2.9 ± 1.0*
Well- to moderately differentiated tumor ( <i>n</i> = 7)	63 ± 13	1.7 ± 0.7
Poorly to undifferentiated tumor ( <i>n</i> = 4)	3 ± 1 <sup>‡</sup>	1.1 ± 0.1
Cells at invasive front ( <i>n</i> = 2)	5 ± 1 <sup>‡</sup>	1.4 ± 0.1

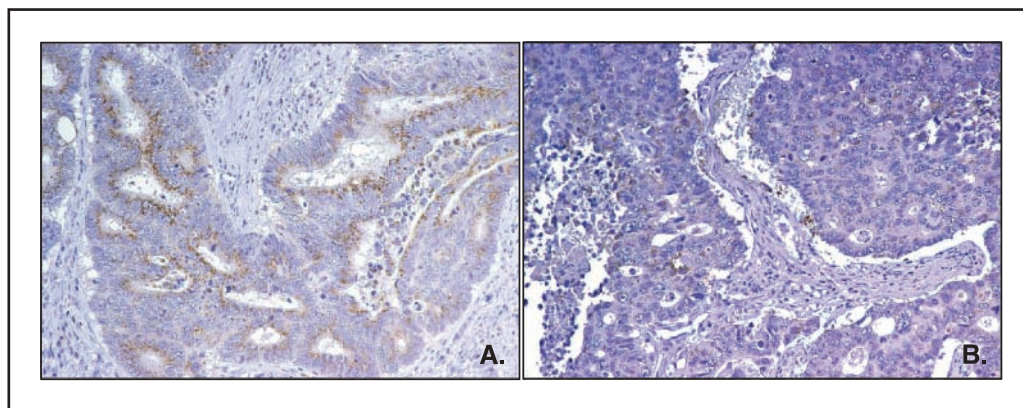
NOTE: Normal colon. Basal zone: base of the crypt containing small, densely packed cells without evidence of goblet cell structure. Mid-crypt: area from the edge of the basal zone to an area 1/3 of the way from the top of the crypt. Surface zone: upper 1/3 of the crypt and the cells at the apex of the crypt. Values are means and standard errors based on four separate areas in each specimen.

Colon carcinoma. Well- to moderately differentiated tumor: evidence of glandular structure. Poorly to undifferentiated tumor: areas in which only cell sheets were detected (i.e., no glandular structure). Invasive front: small clusters of tumor cells (1-3 cells) imbedded in the stroma. Values are means and standard errors based on four separate areas in each specimen.

\**P* < 0.01 statistically significant difference from basal zone.

<sup>†</sup> *P* < 0.05, statistically significant difference from basal zone.

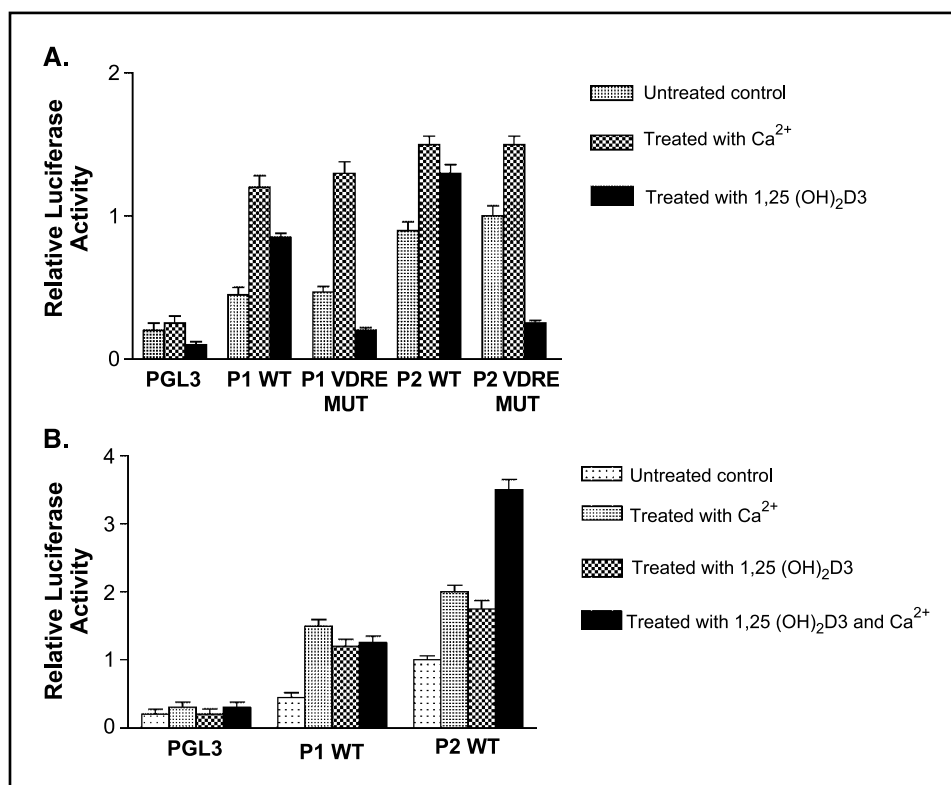
<sup>‡</sup> *P* < 0.01, statistically significant difference from well- to moderately differentiated tumor.



**Figure 2.** CaSR expression in colon carcinomas. *A*, differentiated tumor; *B*, undifferentiated tumor. All sections:  $\times 400$ .

In addition to examining the two agents separately, reporter gene assays were carried out in the presence of both agents. Combination of  $\text{Ca}^{2+}$  and  $1,25(\text{OH})_2\text{D}_3$  did not stimulate P1 over and above the stimulation seen with either alone (Fig. 3*B*). In contrast,  $\text{Ca}^{2+}$  and  $1,25(\text{OH})_2\text{D}_3$  together, stimulated P2 in an additive manner (Fig. 3*B*). These results showed that the *CaSR* gene promoters in these cells are functionally responsive to extracellular  $\text{Ca}^{2+}$  and  $1,25(\text{OH})_2\text{D}_3$ . Elucidation of the mechanisms underlying the differences between the responses of P1 and P2 to the combination of  $\text{Ca}^{2+}$  and VD3 will require further study. Next, we determined if extracellular  $\text{Ca}^{2+}$  and  $1,25(\text{OH})_2\text{D}_3$  could stimulate CaSR expression at the protein level. Immunoblot analyses showed that either  $\text{Ca}^{2+}$  or  $1,25(\text{OH})_2\text{D}_3$  stimulated the expression of CaSR and that a combination of  $\text{Ca}^{2+}$  and  $1,25(\text{OH})_2\text{D}_3$  enhanced the stimulation of CaSR expression (Fig. 4).

Taken together, these results showed that in human colon epithelium-derived CBS cells, either  $\text{Ca}^{2+}$  or  $1,25(\text{OH})_2\text{D}_3$  stimulated CaSR promoter activity and CaSR protein expression. In addition, a combination of  $\text{Ca}^{2+}$  and  $1,25(\text{OH})_2\text{D}_3$  enhanced both CaSR promoter activities and protein expression. We hypothesized that extracellular  $\text{Ca}^{2+}$  and  $1,25(\text{OH})_2\text{D}_3$  are both stimulators of CaSR expression in the normal colonic crypt. It is attractive to speculate that a concentration gradient of  $\text{Ca}^{2+}$  and/or  $1,25(\text{OH})_2\text{D}_3$  exists between the base and apex of the crypt, and that a higher concentration of  $\text{Ca}^{2+}$  and/or  $1,25(\text{OH})_2\text{D}_3$  (to which the colonic epithelium is exposed) at the apex is responsible for CaSR expression and concomitant expression of differentiated cell features. Because the blood  $\text{Ca}^{2+}$  level is tightly controlled (1), the  $\text{Ca}^{2+}$  concentration in the intestinal extracellular fluid may be a critical source of this agonist. Water reabsorption in the colon

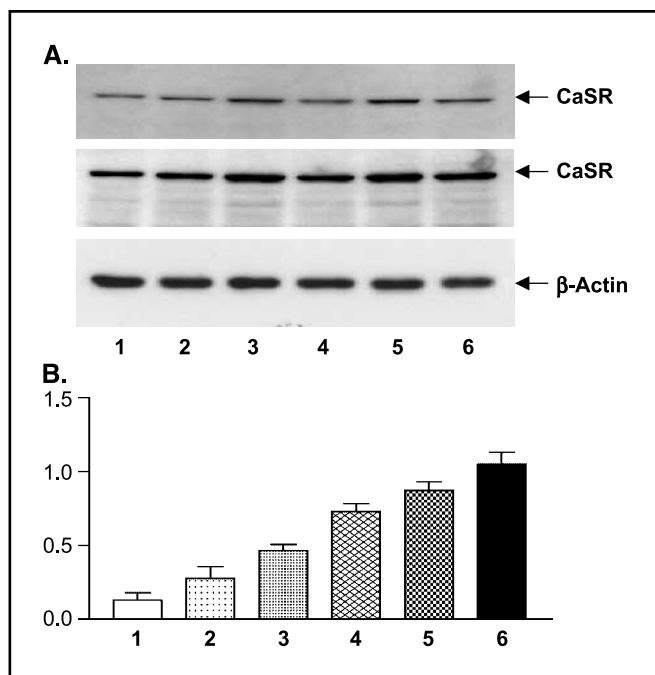


**Figure 3.** Relative transcriptional activity of human CaSR promoters in human colon carcinoma CBS cells. This assay was performed as described in Materials and Methods using previously described reporter constructs (26). *PGL3*, control construct; *P1 WT*, promoter 1 wild type; *P1 VDRE MUT*, P1 promoter with mutated VDRE; *P2 WT*, promoter 2 wild type; and *P2 VDRE MUT*, P2 promoter with mutated VDRE. Columns, means; bar, SE.

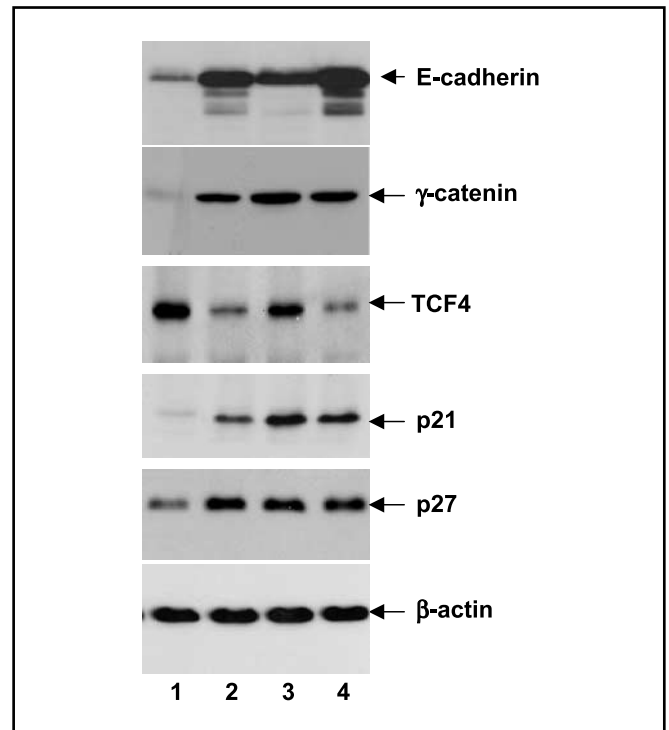


could be expected to increase the Ca<sup>2+</sup> concentration. Likewise, 1,25(OH)<sub>2</sub>D<sub>3</sub> derived from vitamin D in the intestinal fluid may provide the necessary stimulus for differentiation. In a similar fashion, carcinomas with residual glandular structure may be more sensitive to Ca<sup>2+</sup> and/or 1,25(OH)<sub>2</sub>D<sub>3</sub> than undifferentiated tumors, which exist as masses of cells. If this is correct, the implication is that failure to differentiate normally may be a consequence of the malignant growth pattern rather than the cause. Additional experiments will be needed to confirm this and to determine exactly how CaSR expression is regulated in normal and malignant colon epithelium.

Because Ca<sup>2+</sup> and 1,25(OH)<sub>2</sub>D<sub>3</sub> are known to have similar actions (9, 24), we next determined if the two differentiation-inducing agents produced similar downstream effects in CBS cells. The effects of combining Ca<sup>2+</sup> and 1,25(OH)<sub>2</sub>D<sub>3</sub> were also determined. The molecular actions of Ca<sup>2+</sup> and 1,25(OH)<sub>2</sub>D<sub>3</sub> were evaluated in terms of their effects on the expression of E-cadherin,  $\gamma$ -catenin, TCF4, p21, and p27. These markers were chosen because they are functionally associated with the promotion or induction of differentiation in colon carcinomas (19, 27–29). E-cadherin functions in intercellular adhesion and tumor suppression (10–12), whereas the cyclin-dependent kinase inhibitors p21 and p27 function to block cell cycle progression and induce quiescence (28–31). TCF4 is a member of a transcription factor complex involved in the activation of a variety of genes associated with



**Figure 4.** Immunoblot analysis of CaSR expression in the CBS cells in response to Ca<sup>2+</sup> and 1,25(OH)<sub>2</sub>D<sub>3</sub> treatments. Cells were exposed to Ca<sup>2+</sup> or 1,25(OH)<sub>2</sub>D<sub>3</sub> or a combination as described below for 24 hours. *A*, top, short exposure of the membrane (3 minutes of reaction using the chemiluminescence detection method); bottom, long exposure of the membrane (5 minutes of reaction using the chemiluminescence detection method). Membrane was stripped and reprobed with anti- $\beta$  actin antibodies as a control for equal protein loading. *B*, quantitative densitometric analysis of CaSR expression. Columns, ratio of CaSR to  $\beta$ -actin in each lane. Lane 1, untreated control; lane 2, treated with 1 mmol/L Ca<sup>2+</sup>; lane 3, treated with 0.1  $\mu$ mol/L 1,25(OH)<sub>2</sub>D<sub>3</sub>; lane 4, treated with a combination of 1 mmol/L Ca<sup>2+</sup> and 0.1  $\mu$ mol/L 1,25(OH)<sub>2</sub>D<sub>3</sub>; lane 5, treated with 1.0  $\mu$ mol/L 1,25(OH)<sub>2</sub>D<sub>3</sub>; lane 6, treated with 1 mmol/L Ca<sup>2+</sup> and 1.0  $\mu$ mol/L 1,25(OH)<sub>2</sub>D<sub>3</sub>.



**Figure 5.** Immunoblot analysis of the cellular responses to Ca<sup>2+</sup>, 1,25(OH)<sub>2</sub>D<sub>3</sub>, and a combination of Ca<sup>2+</sup> and 1,25(OH)<sub>2</sub>D<sub>3</sub>. Cells were exposed to 1 mmol/L Ca<sup>2+</sup> or 0.1  $\mu$ mol/L 1,25(OH)<sub>2</sub>D<sub>3</sub> or a combination of 1 mmol/L Ca<sup>2+</sup> and 0.1  $\mu$ mol/L 1,25(OH)<sub>2</sub>D<sub>3</sub> for 24 hours. Following treatment, cell extracts were prepared and analyzed by immunoblot for the expression of various molecules related to growth control and differentiation: E-cadherin,  $\gamma$ -catenin, TCF4, and the cyclin-dependent kinase inhibitors p21 and p27. Lane 1, untreated control; lane 2, treated with 1 mmol/L Ca<sup>2+</sup>; lane 3, treated with 0.1  $\mu$ mol/L 1,25(OH)<sub>2</sub>D<sub>3</sub>; lane 4, treated with a combination of 1 mmol/L Ca<sup>2+</sup> and 0.1  $\mu$ mol/L 1,25(OH)<sub>2</sub>D<sub>3</sub>.

malignant cell behavior (23), whereas  $\gamma$ -catenin can bind and inactivate TCF4 (32, 33). Both Ca<sup>2+</sup> and 1,25(OH)<sub>2</sub>D<sub>3</sub> stimulated the expression of E-cadherin and Ca<sup>2+</sup> was more potent (Fig. 5). Combination of Ca<sup>2+</sup> and 1,25(OH)<sub>2</sub>D<sub>3</sub> was more effective than either agent alone in stimulating E-cadherin expression (Fig. 5). Both agents stimulated the expression of  $\gamma$ -catenin and 1,25(OH)<sub>2</sub>D<sub>3</sub> was slightly more potent in stimulating  $\gamma$ -catenin expression (Fig. 5). Both Ca<sup>2+</sup> and 1,25(OH)<sub>2</sub>D<sub>3</sub> stimulated the expression of p21 and p27 and no appreciable enhancement of stimulation was observed using a combination of Ca<sup>2+</sup> and 1,25(OH)<sub>2</sub>D<sub>3</sub> (Fig. 5). 1,25(OH)<sub>2</sub>D<sub>3</sub>, however, seemed to be more potent in stimulating the expression of p21. Interestingly, Ca<sup>2+</sup> but not 1,25(OH)<sub>2</sub>D<sub>3</sub> was found to suppress the expression of TCF4 (Fig. 5). Combining 1,25(OH)<sub>2</sub>D<sub>3</sub> with Ca<sup>2+</sup> did not interfere with the effect of Ca<sup>2+</sup> in suppressing the expression of TCF4 (Fig. 5). This is of interest in light of our recent studies showing a correlation between E-cadherin induction by Ca<sup>2+</sup> and reduced TCF4 activation activity (9). Thus, the induction of E-cadherin by Ca<sup>2+</sup> is linked to the suppression of TCF4 expression and CaSR activation. Because 1,25(OH)<sub>2</sub>D<sub>3</sub> had little effect on the expression of TCF4, it is likely that the intracellular signal pathway of the Ca<sup>2+</sup> and CaSR system leading to the suppression of TCF4 is uniquely associated with this ligand receptor system, and 1,25(OH)<sub>2</sub>D<sub>3</sub> is unable to activate this pathway. The exact nature of this pathway is not known. The relationship between the release of Ca<sup>2+</sup> from intracellular stores due to CaSR activation or due to treatment with 1,25(OH)<sub>2</sub>D<sub>3</sub> on the induction of differentiation

in the CBS cells is also not known and requires further investigation. Interestingly in other cell types such as fibroblasts, raising the extracellular  $\text{Ca}^{2+}$  level results in a sustained increase in intracellular  $\text{Ca}^{2+}$ , whereas agents like  $1,25(\text{OH})_2\text{D}_3$  induce only a transient effect on cytosolic  $\text{Ca}^{2+}$  levels (34). The effects of extracellular  $\text{Ca}^{2+}$  and  $1,25(\text{OH})_2\text{D}_3$  on the kinetics and levels of  $\text{Ca}^{2+}$  release from intracellular stores in the CBS cells are not known. Differential regulation of intracellular  $\text{Ca}^{2+}$  release by extracellular  $\text{Ca}^{2+}$  and  $1,25(\text{OH})_2\text{D}_3$  could be a mechanism in driving different regulatory pathways.

It is concluded that both  $\text{Ca}^{2+}$  and  $1,25(\text{OH})_2\text{D}_3$  induce a more differentiated or less malignant phenotype in colon carcinoma cells. The molecular actions of  $\text{Ca}^{2+}$  and  $1,25(\text{OH})_2\text{D}_3$  seem to be similar in that both agents stimulated CaSR expression and downstream events related to CaSR expression. Whereas the two

agents have similar effects, they are not identical. It thus seems that  $\text{Ca}^{2+}$  and  $1,25(\text{OH})_2\text{D}_3$  affect differentiation and growth control in colon epithelium by influencing multiple mechanisms, some of which intersect at the CaSR. Because there is a strong correlation between CaSR expression and differentiation in colon epithelium, molecular mechanisms involved in regulating CaSR expression and function seem to be critical in regulating epithelial growth and differentiation in the colon.

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## References

- Brown EM, Pollak M, Hebert SC. The extracellular calcium-sensing receptor: its role in health and disease. *Annu Rev Med* 1998;49:15–29.
- Hebert SC, Brown EM. The extracellular calcium receptor. *Curr Opin Cell Biol* 1995;7:484–92.
- McNeil SE, Hobson SA, Nipper V, Rodland K. Functional calcium-sensing receptors in rat fibroblasts are required for activation of *src* kinase and mitogen-activated protein kinase in response to extracellular calcium. *J Biol Chem* 1998;273:1114–20.
- Rutten MJ, Bacon KD, Marlink KL, et al. Identification of a functional  $\text{Ca}^{2+}$ -sensing receptor in normal human gastric mucous epithelial cells. *Am J Physiol* 1999;277:G662–70.
- Tu C-L, Oda Y, Bikle DD. Effects of a calcium receptor activator on the cellular response to calcium in human keratinocytes. *J Invest Dermatol* 1999;113:340–5.
- Lipkin M. Preclinical and early human studies of calcium and colon cancer prevention. *Ann NY Acad Sci* 1999;889:120–7.
- Wargovich MJ, Jimenez A, McKee K, et al. Efficacy of potential chemopreventive agents on rat colon aberrant crypt formation and progression. *Carcinogenesis* 2000;21:1149–55.
- Sheinin Y, Kallay E, Wrba F, Kriwanek S, Peterlik M, Cross HS. Immunocytochemical localization of the extracellular calcium-sensing receptor in normal and malignant human large intestinal mucosa. *J Histochem Cytochem* 2000;48:595–601.
- Chakrabarty S, Radjendirane V, Appelman H, Varani J. Extracellular calcium and calcium sensing receptor function in human colon carcinomas: promotion of E-cadherin expression and suppression of  $\beta$ -catenin/TCF activation. *Cancer Res* 2003;63:67–71.
- Takeichi M. Cadherin cell adhesion receptors as a morphogenetic regulator. *Science* 1991;251:1451–5.
- Gumbiner BM, McCreia PD. Catenins as mediators of the cytoplasmic functions of cadherins. *J Cell Sci* 1993;17:155–8.
- Valizadeh A, Karayiannakis AJ, El-Hariry I, Kmiot W, Pignatelli M. Expression of E-cadherin-associated molecules ( $\alpha$ -,  $\beta$ , and  $\gamma$ -catenins and p120) in colorectal polyps. *Am J Pathol* 1997;150:1977–84.
- Kinsella AR, Lepts GC, Hill CL, Jones M. Reduced E-cadherin expression correlates with increased invasiveness in colorectal carcinoma cell lines. *Clin Exp Metastasis* 1994;12:335–42.
- MacCalman CD, Brodt P, Doublet JD, et al. The loss of E-cadherin transcripts in rat prostate tumors is accompanied by increased expression of mRNA transcripts encoding fibronectin and its receptor. *Clin Exp Metastasis* 1994;12:101–7.
- Watabe M, Nagafuchi A, Tsukita S, Takeichi M. Induction of polarized cell-cell association and retardation of growth by activation of the E-cadherin-catenin adhesion system in a dispersed carcinoma line. *J Cell Biol* 1994;127:247–56.
- Dorudi S, Hanby AM, Poulson R, Northover J, Hart IR. Level of expression of E-cadherin mRNA in colorectal cancer correlates with clinical outcome. *Br J Cancer* 1995;71:614–6.
- Jawhari A, Farthing M, Pignatelli M. The importance of E-cadherin-catenin complex in the maintenance of intestinal epithelial homeostasis: more than intercellular glue? *Gut* 1997;41:581–4.
- Breen E, Steele G, Mercurio AM. Role of the E-cadherin/ $\alpha$ -catenin complex in modulating cell-cell and cell-matrix adhesive properties of invasive colon carcinoma cells. *Ann Surg Oncol* 1995;2:378–85.
- Aken EV, De Wever O, Correia da Rocha AS, Mareel M. Defective E-cadherin/catenin complexes in human cancer. *Virchows Arch* 2001;439:725–51.
- Peifer M. Cell adhesion and signal transduction: the Armadillo connection. *Trends Cell Biol* 1995;5:224–9.
- Moon RT, Brown JD, Torres M. WNTs modulate cell fate and behavior during vertebrate development. *Trends Genet* 1997;13:157–62.
- Sharp C, Lawrence N, Arias AM. Wnt signalling: a theme with nuclear variations. *BioEssays* 2001;23:311–8.
- Behrens J, Jerchow B-A, Wurtele M, et al. Functional interaction of an axin homolog, conductin, with  $\beta$ -catenin, APC and GSK3 $\beta$ . *Science* 1998;280:596–9.
- Palmer HG, Gonzalez-Sancho JM, Espada J, et al. Vitamin D3 promotes the differentiation of colon carcinoma cells by the induction of E-cadherin and the inhibition of  $\beta$ -catenin signaling. *J Cell Biol* 2001;154:369–77.
- Laaksonen M, Karkkainen M, Outila T, Vanninen T, Ray C, Lamborg-Allart C. Vitamin D receptor gene *BsmI*-polymorphism in Finnish premenopausal and postmenopausal women: its association with bone mineral density, marker of bone turnover, and intestinal calcium absorption, with adjustment for lifestyle factors. *J Bone Miner Metabol* 2002;20:383–90.
- Canaff L, Hendy GN. Human calcium-sensing receptor gene. Vitamin D response elements in promoters P1 and P2 confer transcriptional responsiveness to 1,25-dihydroxyvitamin D. *J Biol Chem* 2002;277:30337–50.
- Wang H, Chakrabarty S. Requirement of protein kinase C $\alpha$ , extracellular matrix remodeling and cell-matrix interaction for transforming growth factor  $\beta$ -regulated expression of E-cadherin catenins. *J Cell Physiol* 2001;187:188–95.
- Mariadson JM, Bordonaro M, Aslam F, et al. Down-regulation of  $\beta$ -catenin TCF signaling is linked to colonic epithelial cell differentiation. *Cancer Res* 2001;61:3465–71.
- Steinman RA, Hoffman B, Iro A, Guilouf C, Liebermann DA, El-Houseini ME. Induction of p21(WAF-1/CIP1) during differentiation. *Oncogene* 1994;9:3389–96.
- Hartwell LH, Kastan MB. Cell cycle control and cancer. *Science* 1994;266:1821–8.
- Jacks T, Weinberg RA. The expanding role of cell cycle regulators. *Science* 1998;280:1035–7.
- Winn RA, Bremnes RM, Bemis L, et al.  $\gamma$ -Catenin expression is reduced or absent in a subset of human lung cancers re-expression inhibits transformed cell growth. *Oncogene* 2002;21:7497–506.
- Moreno-Bueno G, Hardisson D, Sarrío D, et al. Abnormalities of E- P- cadherin and catenin ( $\beta$ -,  $\gamma$ -catenin, and p120 ctn) expression in endometrial cancer and endometrial atypical hyperplasia. *J Pathol* 2003;199:471–8.
- Varani J, Shayevitz J, Perry D, Mitra RS, Nickoloff BJ, Voorhees J. Retinoic acid stimulation of human dermal fibroblast proliferation is dependent on suboptimal extracellular  $\text{Ca}^{2+}$  concentration. *Am J Pathol* 1990;136:1275–81.

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## Calcium Sensing Receptor in Human Colon Carcinoma: Interaction with $\text{Ca}^{2+}$ and 1,25-Dihydroxyvitamin $\text{D}_3$

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