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### ► To cite this version:

Aurélie Theillaumas, Martine Blanc, Christophe Couderc, Gilles Poncet, Wissam Bazzi, et al.. Relation between menin expression and NF- $\kappa$ B activity in an intestinal cell line. *Molecular and Cellular Endocrinology*, 2008, 291 (1-2), pp.109. 10.1016/j.mce.2008.05.019 . hal-00532032

**HAL Id: hal-00532032**

**<https://hal.science/hal-00532032>**

Submitted on 4 Nov 2010

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## Accepted Manuscript

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PII: S0303-7207(08)00245-1  
DOI: doi:10.1016/j.mce.2008.05.019  
Reference: MCE 6889

To appear in: *Molecular and Cellular Endocrinology*

Received date: 18-2-2008  
Revised date: 28-4-2008  
Accepted date: 27-5-2008

Please cite this article as: Theillaumas, A., Blanc, M., Couderc, C., Poncet, G., Bazzi, W., Bernard, C., Cordier-Bussat, M., Scoazec, J.Y., Roche, C., Relation between menin expression and NF- $\kappa$ B activity in an intestinal cell line, *Molecular and Cellular Endocrinology* (2007), doi:10.1016/j.mce.2008.05.019

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**Relation between menin expression and NF- $\kappa$ B activity in an intestinal cell line.**

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**Abstract**

In a previous study, we demonstrated that the *Men1* gene is mainly expressed in the proliferative crypt compartment of the small intestine and that a reduction of menin expression in the crypt-like IEC-17 cell line induces an increase in proliferation rate concomitant with an increase in cyclin D1 expression. The aim of the present study was to test the hypothesis that the NF- $\kappa$ B pathway may be involved in cyclin D1 overexpression. Transcriptional activity of the cyclin D1 gene promoter was increased upon reduction of menin expression. Blockade of the NF- $\kappa$ B pathway restored proliferation, cell cycle, cyclin D1 gene transcription and cyclin D1 expression levels to those observed in the presence of menin. These data support a correlation between cyclin D1 expression, NF- $\kappa$ B activity and menin expression in this epithelial cell line and are relevant to the physiological function of menin in regulating proliferation in the intestinal epithelium.

Keywords: menin, cyclin D1, NF- $\kappa$ B, proliferation, endocrine tumor

## 1. Introduction

Neuroendocrine tumors of the gastrointestinal tract constitute a group of uncommon and heterogeneous neoplasms. Some of them occur in a genetic context, mainly represented by the mutations of the tumor suppressor gene *MEN1*. This gene encodes an original protein of unknown biochemical function called menin and is mutated in the Multiple Endocrine Neoplasia type 1 (MEN1) syndrome. Despite the endocrine specificity of the MEN1 syndrome tumors (parathyroids, pituitary, duodeno-pancreas), menin is expressed in almost all normal tissues and plays a role in the regulation of various cellular functions such as gene transcription, cell proliferation, apoptosis and genome stability (Yang and Hua, 2007). One of the best studied functions is the role of menin in regulating cellular proliferation: menin overexpression slows proliferation of RAS-transformed NIH3T3 cells and suppresses tumorigenesis (Kim et al., 1999). MEF obtained from homozygous *Men1*<sup>-/-</sup> mice proliferate more rapidly than wild-type cells while re-expressing menin in the MEF *Men1*<sup>-/-</sup> cells restores normal cell proliferation rate (Schnepf et al., 2004). Several recent reports show that menin directly regulates expression of the cyclin-dependent kinase inhibitors p27<sup>kip1</sup> and p18<sup>ink4c</sup> (Karnik et al., 2005; Milne et al., 2005). Furthermore, *in vivo* *Men1* excision enhances cell proliferation in pancreatic islets *in vivo*, through an accelerated S-phase entry (Schnepf et al., 2006). Interestingly, a high tissue expression of menin often correlates with the distribution of the proliferative compartment, such as in the endometrium and in the oesophageal mucosa (Ikeo et al., 2000). The intestinal epithelium is one of the most rapidly proliferating epithelia in the organism and in a previous study we show by *in situ* hybridization that the *Men1* gene is mainly expressed in the proliferative cryptic compartment of the small intestine (Ratineau et al., 2004).

The IEC-17 cell line is a non-transformed crypt-like cell line representative of the proliferative compartment of the small intestine. By reducing the endogenous expression of

menin through an antisense strategy in this cell line, we induced an increase in proliferation rate, an increase in cyclin D1 protein level and the tumorigenic transformation of these cells (Ratineau et al., 2004). Cyclin D1 is a key cell cycle regulatory protein and constitutive expression of cyclin D1 is a frequent abnormality observed in human cancer leading to enhanced cell transformation and tumorigenesis (Fu et al., 2004). Cyclin D1 expression is regulated at multiple levels, one of them being the transcriptional regulation of the cyclin D1 gene. The cyclin D1 promoter contains a LEF-1 binding sequence which is activated by the  $\beta$ -catenin/LEF-1 complex in colon cancer cells (Shtutman et al., 1999). However we demonstrated that a deregulation of this pathway was not responsible for the cyclin D1 overexpression observed in the low-menin expressing clones of the IEC-17 cell line (Ratineau et al., 2004). Several NF- $\kappa$ B binding sites have been clearly identified in the cyclin D1 gene promoter and are involved in the control of cell growth (Guttridge et al., 1999). Furthermore, menin has been reported to interact with NF- $\kappa$ B proteins and to inhibit the NF- $\kappa$ B-mediated transcriptional activation (Heppner et al., 2001). An NF- $\kappa$ B activation has been reported in several human neoplasias including breast cancers, prostate tumors, melanoma, hepatocellular carcinomas and pancreatic cancer (Dolcet et al., 2005).

We thus hypothesized that the cyclin D1 overexpression observed in the low-menin expressing IEC-17 cells might be due to a transcriptional activation of the cyclin D1 gene through a NF- $\kappa$ B dependent mechanism. The aim of the present study was to investigate the relation between menin expression and transcriptional activity of the cyclin D1 gene, then to evaluate the effect of NF- $\kappa$ B inhibition in a low-menin expressing clone of the IEC-17 cell line.

## 2. Methods

### 2.1. Materials

The anti-menin antibody (BL342) was obtained from Bethyl Laboratories, the anti-cyclin D1 (sc 8396), anti-NF- $\kappa$ B p65 (sc-372), anti-TFIIHp89 (sc-293) antibodies were obtained from Santa Cruz Biotechnology and the anti-phospho-NF- $\kappa$ B p65 (3037) was obtained from Cell Signaling Technology. The anti  $\alpha$ -tubulin (B5-1-2) antibody was purchased from Sigma-Aldrich. The pharmacological inhibitors parthenolide and curcumin were obtained respectively from Santa Cruz Biotechnology and Sigma-Aldrich.

### 2.2. Cell culture

Stable clones of the rat duodenal cell line IEC-17 were obtained after transfection of an expression plasmid encoding the human menin cDNA cloned in an antisense orientation as previously described (Ratineau et al., 2004). The control clone V403 containing the empty vector and the antisense clone AS303 used in this study were routinely maintained in DMEM supplemented with 10%FCS, 25 units/ml insulin, 2mM glutamine, 100 units/ml penicillin, 50mM streptomycin and 700 $\mu$ g/ml G418. Mouse Embryonic Fibroblasts (MEF) derived from homozygotes *Men1*<sup>-/-</sup> mouse embryos were a generous gift from CX Zhang (CNRS UMR 5201, Lyon, France). These cells were cultured in DMEM, 10%FCS, 2mM glutamine, 100 units/ml penicillin, 50mM streptomycin and 100 $\mu$ M 2-mercapto-ethanol.

### 2.3. Transient transfections and luciferase assays

The cyclin D1 luciferase reporter plasmid -1745CD1-LUC and the expression plasmid encoding the human menin cDNA cloned into the EcoRI site of pcDNA3.1 were generous gifts from RG Pestell (Georgetown University School of Medicine, Washington DC) and GN Hendy (Mc Gill University, Montreal, Canada), respectively. The expression plasmid containing a dominant-negative acting I $\kappa$ B $\alpha$  mutant (IkBM) was obtained from Clontech (Ozyme, Saint Quentin en Yvelines, France). Transient transfections were performed by

seeding 50,000 cells per well in 24-well plates for MEF<sup>-/-</sup> or 70,000 cells per well in 12-well plates for V403 and AS303 and growing the cells in complete medium for 24 h. The following day, cells were transfected with a total of 1 $\mu$ g (for MEF<sup>-/-</sup>) or 2 $\mu$ g (for V403 and AS303) of DNA plasmid and a 1:100 amount of the phRL-TK plasmid expressing Renilla using jetPEI as recommended by the manufacturer (Polyplus-transfection, Illkirch, France). Cells were harvested 48 hours later; luciferase and renilla activities were monitored using the dual-luciferase reporter assay system (Promega, Charbonnières, France).

#### *2.4. Protein extraction and Western blot analysis*

Proteins were either extracted using a Whole Cell Extraction Kit or a Nuclear Extraction Kit (Chemicon-Millipore, Saint Quentin en Yvelynes, France), according to the manufacturer's instructions. Cell lysates were resolved on 7.5-10% SDS polyacrylamide gel and blotted to PVDF membranes (Millipore) as previously described (Ratineau et al., 2004). For menin detection, cell lysates were resolved on 8% bis acrylamide gel and protein transfer on PVDF membrane was performed in CAPS buffer (Cyclohexylaminopropanesulfonic acid 10mM, 10% methanol). Saturation was achieved in 0.1% Tween in Tris-buffered saline with 5% milk for 1 hour. The membranes were probed with primary antibodies overnight at 4°C, washed and incubated with appropriate immunoperoxidase-conjugated secondary antibodies (Jackson ImmunoResearch Laboratories) for 3 hours at 4° C (1 hour at room temperature for menin). Antibody binding was detected using ECL Western Blotting Detection System (Covalab, Villeurbanne, France). Signals were quantified using Image J software.

#### *2.5. MTT cell viability assay*

Cells were seeded at a density of 750 cells /well in 96-well plates and maintained 72 hours in normal culture conditions. Pharmacological inhibitors parthenolide (10 $\mu$ M) and curcumin (20 $\mu$ M) were then added for 24 hours. A 20 $\mu$ l sample of MTT solution ((4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide, 5mg/ml) was added to each well and the plates were



incubated at 37°C for 2.5 hours. The supernatant was then discarded and 100µl DMSO was added to dissolve formazan crystals, generating a blue-purple color. The absorbance was measured at 540nm.

### *2.6. Cell cycle analysis*

Cells in growth phase were synchronized by exposing the culture to FCS-deprived DMEM supplemented with 0.1% BSA for 48 hours. Parthenolide (10µM) was added to the cells for the last 24 hours of the synchronization period. Cells were harvested, washed and the nuclei were isolated and stained with propidium iodide using the BD Cycle TEST™ PLUS Reagent Kit (BD Biosciences, Le Pont de Claix, France) according to the manufacturer's instructions. Flow cytometry was then performed using a DAKO "Galaxy" apparatus.

### *2.7. NF-κB transcription factor DNA binding activity*

Cells were serum starved in DMEM supplemented with 0.1% BSA for 48 hours and parthenolide 10µM was added to the cells for the last 24 hours. Nuclear protein extracts were obtained using a Nuclear Extract Kit (Active Motif, Belgium) according to the manufacturer's instructions. Activation of the NF-κB p65 subunit in the nuclear extracts was measured using an NF-κB p65 ELISA-based transcription factor assay kit (TransAM assay) (Active Motif). Nuclear extracts were incubated with oligonucleotide containing an NF-κB consensus binding site immobilized on 96-well plate. The primary antibody used to detect NF-κB is specific for the active form of p65 from human, mouse and rat origin. Subsequent incubation with an HRP-conjugated secondary antibody provides a sensitive colorimetric readout that is quantified by a spectrophotometer at 450 nm with a reference wavelength of 655 nm.

### *2.8. Immunohistochemistry*

For immunocytochemical study, cells were grown in 8-well chamber slides, then serum starved in DMEM supplemented with 0.1% BSA for 48 hours. Cells were fixed with cold methanol and incubated for 1hour with the rabbit anti-phospho-NF-κB p65 antibody (1:50).

Antigen-antibody complexes were revealed by the streptavidin-biotin technique (Vectastain ABC kit, Vector) using diaminobenzidine as chromogen. Slides were counterstained with hematoxylin. For immunohistochemical study, sections from paraffin-embedded tumors obtained after subcutaneous injection of AS303 cells in immunodepressed newborn rats (Ratineau et al., 2004) were dewaxed, rehydrated, then the endogenous peroxidase was blocked by incubation for 20min with 2% hydrogen peroxide in PBS. Antigen retrieval was achieved by three 5-min microwave cycles in sodium citrate (pH 6.0). Sections were then incubated with the rabbit polyclonal antibody against NF-kB p65 (1:500) for 1 hour at room temperature and the antigen-antibody detection was performed as described above.

### *2.9. Statistical analysis*

Results are presented as mean +/- SEM. Significant differences were analyzed by using the Mann-Whitney test.  $p < 0.05$  was required for statistical significance.

### 3. Results

#### 3.1. Transcriptional activity of the cyclin D1 gene promoter is dependent on menin expression

Our previous study demonstrated that menin inhibition in AS303 cells resulted in a strong overexpression of cyclin D1 at the protein level (Ratineau et al., 2004). The first step of this study was to measure the transcriptional activity of the cyclin D1 gene promoter in V403 and AS303 cells transiently transfected with the -1745CD1-LUC reporter plasmid. We observed that the transcriptional activity of the cyclin D1 gene promoter was higher in AS303 cells than in V403 cells (5.72 +/- 0.37 vs 1.58 +/- 0.29, RLA), correlated to a low menin expression in AS303 cells (Fig 1A). This result suggested that modifications of menin expression might result in up- or down-regulation of the cyclin D1 gene. Several data came in support of this hypothesis. Firstly, we used fibroblasts derived from homozygotes *Men1*<sup>-/-</sup> mouse (MEF<sup>-/-</sup>) that did not express menin. Transient transfection with increasing concentration of the pcDNA3.1/men1 expression vector induced a strong dose-dependent decrease of the transcriptional activity of the cyclin D1 gene promoter, concomitant with the dose-dependent re-expression of the protein menin in these cells (Fig 1B). Accordingly, transient transfection of the pcDNA3.1/men1 vector in V403 cells resulted in a significant decrease of the transcriptional activity of the cyclin D1 gene promoter, whereas it did not occur in AS303 cells, due to the presence of the antisense Men1 cDNA that prevent menin overexpression (Fig 1C). Taken together, these results showed that transcriptional activity of the cyclin D1 gene promoter is dependent on the level of menin expression.

#### 3.2. NF- $\kappa$ B regulates transcriptional activity of the cyclin D1 gene promoter

The transcription factor NF- $\kappa$ B is known to regulate cyclin D1 gene promoter activity (Guttridge et al., 1999). To investigate whether this mechanism occurred in our cellular model, the consequence of NF- $\kappa$ B pathway inhibition was assessed on the cyclin D1 gene promoter activity. For this experiment, this inhibition was achieved by using an expression

plasmid encoding a dominant-negative acting I $\kappa$ B $\alpha$  mutant (IkBM) cotransfected with the -1745CD1-LUC reporter plasmid. The transcriptional activity of the cyclin D1 gene promoter was not modified in V403 cells but was significantly reduced in AS303 cells to a level similar to those observed for V403 cells (Fig 2). These results supported a transcriptional control of the cyclin D1 gene expression by the NF- $\kappa$ B pathway in our cellular model.

### *3.3. Effects of the inhibition of NF- $\kappa$ B in AS303 cells:*

The above results prompted us to test the effects of the inhibition of the NF- $\kappa$ B pathway on the growth properties of the low-menin expressing AS303 cells. For this purpose, two natural inhibitors of the NF- $\kappa$ B pathway, parthenolide and curcumin were used. We performed MTT assays to assess the number of viable cells after a 24-hour exposure to the inhibitors. As shown in figure 3A, AS303 cells grew 3.4 times more rapidly than V403 cells in normal culture conditions. A 24-hour exposure of the AS303 cells to parthenolide (10 $\mu$ M) completely inhibited this accelerated proliferation whereas curcumin (20 $\mu$ M) also significantly reduced it. As parthenolide was more effective than curcumin in reducing the proliferation rate of AS303 cells to those of V403 cells observed in the absence of the drugs, this inhibitor was used for further experiments.

To strengthen the above result, we tested the hypothesis that inhibition of NF- $\kappa$ B pathway with parthenolide should modify cell cycle in the low-menin expressing AS303 cells. For this purpose, V403 and AS303 cells were synchronized by serum starvation for 48 hours and parthenolide (10 $\mu$ M) was added during the last 24 hours of this synchronization period. As we previously described (Ratineau et al., 2004), down regulation of menin expression in AS303 cells had dramatic consequences on cell cycle. Indeed, 18 % of AS303 cells remained in S phase whereas 81% of V403 cells were arrested in G0/G1 phase and only 8% entered S phase. Blockade of NF- $\kappa$ B pathway after treatment with parthenolide of the AS303 cells restored the control of the cell cycle in these cells: 82% were arrested in G0/G1 phase, whereas only 4%

entered S phase (Fig 3B). These data suggested a correlation between menin expression level and NF- $\kappa$ B pathway.

As parthenolide inhibited proliferation in AS303 cells, its effect was thus tested on the expression of cyclin D1. As shown in figure 3C, parthenolide (10 $\mu$ M) did not modify cyclin D1 expression in V403 cells, whereas it significantly suppressed the cyclin D1 overexpression in AS303 cells.

All these results showed that blockade of NF- $\kappa$ B pathway in AS303 cells resulted in a decrease of cyclin D1 gene promoter activity, a decrease of cyclin D1 protein level and a decrease of cell proliferation.

#### *3.4. Active NF- $\kappa$ B p65 subunit increases in AS303 cells*

Taken together, the results strongly suggested an activation of the NF- $\kappa$ B pathway in the low-menin expressing AS303 cells. To gain further insight into this hypothesis, the DNA binding activity of p65 subunit of the NF- $\kappa$ B complex was compared in nuclear extracts from V403 and AS303 cells treated with parthenolide (10 $\mu$ M) using a colorimetric ELISA-based assay. As shown in figure 4A, a strong decrease of the p65 binding activity was observed in AS303 cells in the presence of the inhibitor, whereas only a slight decrease occurred in V403 cells. These results were confirmed at the protein level by Western blot (Fig 4B). Indeed, the nuclear expression of p65 was significantly increased in AS303 cells and also significantly reduced by parthenolide treatment. Interestingly, the total p65 expression remained stable, suggesting NF- $\kappa$ B pathway activation only in AS303 cells. To confirm the nuclear localization of p65 in AS303 cells, the immunoreactivity of the phosphorylated form of p65 was investigated using a specific anti-phospho-NF- $\kappa$ B p65 antibody. As shown in figure 5 A and B, nuclear immunoreactivity was only seen in AS303 cells. Furthermore, p65 immunoreactivity was investigated on sections from an AS303-subcutaneous tumor (Ratineau

et al., 2004): numerous tumor cells exhibited a strong nuclear staining, consistent with the nuclear translocation of the phosphorylated NF- $\kappa$ B p65 protein (Fig 5C).

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#### 4. Discussion

In the present study, we firstly provided data supporting a negative correlation between menin expression and cyclin D1 gene activity in our IEC-17 cellular model. Indeed, the transcriptional activity of the cyclin D1 gene promoter was more than 3 times higher in low menin-expressing AS303 cells than in V403 cells, suggesting that the cyclin D1 gene was regulated at the transcriptional level. The re-expression of menin in MEF obtained from homozygous *Men1*<sup>-/-</sup> mouse embryos or in V403 cells strongly reduced the transcriptional activity of the cyclin D1 gene promoter. Noteworthy, the presence of the antisense *Men1* cDNA in AS303 cells prevented the reduction of transcriptional activity induced by menin overexpression, confirming the efficiency of the antisense strategy in this cell line. The transcriptional activity of the cyclin D1 gene was thus dependent on the menin expression level.

Several factors are able to act as transcriptional regulators of the cyclin D1 gene, two of them being  $\beta$ -catenin/LEF-1 pathway (Shtutman et al., 1999) and NF- $\kappa$ B pathway (Guttridge et al., 1999). We have previously demonstrated the  $\beta$ -catenin/LEF-1 pathway was not deregulated in our low-menin expressing cellular model (Ratineau et al., 2004), which is in keeping with recent immunohistochemical studies showing an infrequent nuclear accumulation of  $\beta$ -catenin in large series of gastroenteropancreatic endocrine tumors (Hervieu et al., 2006; Audard et al., 2008), therefore suggesting the absence of involvement of the Wnt pathway in these tumors. The most probable hypothesis was therefore that an NF- $\kappa$ B dependent mechanism may be involved. One possibility to test this suggestion was to inhibit this pathway. In the cytoplasm, NF- $\kappa$ B and I $\kappa$ B proteins are linked in an inactive complex. Upon activation, I $\kappa$ B proteins are phosphorylated through I $\kappa$ B kinase, targeting them for ubiquitination and proteasomal degradation, leading to the release and nuclear translocation of active NF- $\kappa$ B proteins (Kim et al., 2006). Blockade of this mechanism was here achieved by using a non-phosphorylatable

dominant-negative I $\kappa$ B mutant, which prevents the [I $\kappa$ B]-[NF- $\kappa$ B] complex dissociation, or two natural inhibitors of NF- $\kappa$ B proteins parthenolide and curcumin. Parthenolide is a plant-derived sesquiterpene lactone whereas curcumin is a derivative of the spice turmeric (*Curcuma longa*). Both of them are widely used to inhibit the DNA binding of NF- $\kappa$ B proteins as well as the I $\kappa$ B kinase in cellular or animal tumor models (Oka et al., 2007; Kishida et al., 2007; Lin et al., 2007; Marin et al., 2007). First of all, we demonstrated that inhibition of the NF- $\kappa$ B pathway resulted in the normalization of the transcriptional activity of the cyclin D1 gene promoter in the low-menin expressing AS303 cells. Then, we investigated the effects of NF- $\kappa$ B pathway inhibition on cell proliferation. Parthenolide and curcumin exhibited antiproliferative effect on AS303 cells. In agreement with this observation, parthenolide was able to normalize the cell cycle in AS303 cells, by arresting them in G0/G1 phase. Finally, the overexpression of cyclin D1 observed in AS303 cells was reduced upon incubation with parthenolide. Taken together, the data provided here showed that cyclin D1 overexpression observed in low-menin expressing AS303 cells resulted from transcriptional activation of the cyclin D1 gene through an NF- $\kappa$ B dependent mechanism.

The last step of our study was to provide evidence that NF- $\kappa$ B pathway was activated in the low-menin expressing AS303 cells. This was done by measuring the DNA binding capacity of the p65 subunit of the NF- $\kappa$ B protein complex, which was higher in AS303 cells and inhibited by parthenolide. Accordingly, a positive nuclear immunoreactivity for the phosphorylated form of p65 was observed in AS 303 cells.

Taken together, our observations allow us to propose a correlation between menin expression, cyclin D1 expression and NF- $\kappa$ B pathway in our intestinal epithelial cell model. As suggested in Fig 6, the level of menin expression regulates the amount of active proteins of the NF- $\kappa$ B complex available to interact with the cyclin D1 gene. Interestingly, this is relevant to the physiological functions of menin as well as its role in tumor formation. Concerning the



physiological functions, our present study supports previous results demonstrating the same cryptic localization of menin and cyclin D1 (Ratineau et al., 2004; Chandrasekaran et al., 1996) and the involvement of NF- $\kappa$ B in the regulation of colonic epithelial cell turnover (Inan et al., 2000). A physiological function for menin within the cryptic intestinal compartment may be to take part in regulating proliferation and prevent an uncontrolled increase in the number of intestinal epithelial cells. Moreover, since the reduction of menin expression in IEC-17 cell line leads to the tumorigenic transformation of these cells, our data are also relevant to the role of menin in tumor formation. Indeed, physiopathologic disorders induced by the abnormalities of menin expression mainly concern endocrine tumors in the duodeno-pancreatic area, parathyroids and pituitary. In these particular tumors, the relation between menin expression and cyclin D1 or NF- $\kappa$ B activity is not well documented. Overexpression of cyclin D1 frequently occurs in pancreatic endocrine tumors (Chung et al., 2000; Guo et al., 2003) or in parathyroid adenomas (Mallya et al., 2000), and this overexpression has been postulated to be an early event in neuroendocrine tumorigenesis (Chung, 2004). Parathyroid tumorigenesis is dependent on both cyclin D1 and menin (Rosenberg et al., 1991; Bhuiyan et al., 2000) and parathyroid hyperplasia is induced by an overexpression of cyclin D1 or a loss of menin function in transgenic mice (Imanishi et al., 2001; Bertolino et al., 2003; Libutti et al., 2003). Furthermore, a negative correlation between menin expression and p65 phosphorylation was demonstrated in human parathyroid tumors, as well as a decrease in cyclin D1 expression in response to inhibition of the NF- $\kappa$ B pathway (Corbetta et al., 2005). The data we provided here are in accordance with these latter findings. However, such a correlation has not been established so far in gastroenteropancreatic neuroendocrine tumors. It thus become very interesting to investigate the activity and function of the NF- $\kappa$ B pathway in relation to menin and cyclin D1 expression in these tumors by means of suitable endocrine cellular models which are in progress in our lab. All these studies underline the interest of NF-

kB pathway as therapeutic target in the treatment of neuroendocrine tumors, and generally, a better understanding of the intracellular mechanisms affected by a deregulation of menin expression constitutes a promising challenge for the identification of new therapeutic targets.

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**Acknowledgments**

We acknowledge the CeCIL (Centre Commun d'Imagerie Laennec) for providing part of the technical devices used in this work.

This work was supported by grants from the Comité de la Drôme de la Ligue contre le Cancer to C Roche and Fondation pour la Recherche Médicale to A Theillaumas.

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## FIGURE LEGENDS

Fig.1: Transcriptional activity of the cyclin D1 gene promoter is dependent of menin expression. A, V403 and AS303 cells were transfected for 48h with the cyclin D1 luciferase reporter plasmid -1745CD1-LUC (1  $\mu$ g, denoted pD1). \*  $p < 0.05$  vs pD1 transcriptional activity in V403 cells. Menin expression was assessed by Western-blot analysis using a specific anti-menin antibody and the anti  $\alpha$ -tubulin antibody as equal loading control. B, MEF<sup>-/-</sup> cells were cotransfected for 48h with the -1745CD1-LUC plasmid (0.25 $\mu$ g, pD1) and increasing concentrations of the pcDNA3.1/men1 vector (Men1). The dose-dependent re-expression of the protein menin was checked by Western-blot, using a specific anti-menin antibody and the anti-TFIIHp89 antibody as equal loading control (p89). \*  $p < 0.05$  vs pD1 transcriptional activity in the absence of menin. C, V403 and AS303 cells were cotransfected for 48h with the -1745CD1-LUC plasmid (1 $\mu$ g, pD1) and increasing concentrations of the pcDNA3.1/men1 vector (Men1). \*  $p < 0.05$  vs pD1 transcriptional activity in the absence of menin reexpression. Transfection experiments results are expressed as RLA (Relative Luciferase Activity) after normalization of the Luciferase activity to the relative Renilla activity in each experiment. The data are plotted as the mean  $\pm$  SE of four independent experiments performed in triplicate.

Fig.2. NF- $\kappa$ B regulates transcriptional activity of the cyclin D1 gene promoter. V403 and AS303 cells were transfected for 48h with the -1745CD1-LUC plasmid (1 $\mu$ g, pD1), and an expression plasmid encoding a dominant-negative acting I $\kappa$ B $\alpha$  mutant (1 $\mu$ g, I $\kappa$ BM). Results are expressed as RLA (Relative Luciferase Activity) after normalization of the Luciferase activity to the relative Renilla activity in each experiment and plotted as mean  $\pm$  SD of three independent experiments performed in quadruplicate. §  $p < 0.05$  vs pD1 transcriptional activity in V403 cells, \*  $p < 0.05$  vs pD1 transcriptional activity in the absence of parthenolide.

Fig.3. Effects of the inhibition of the NF- $\kappa$ B pathway. A, AS303 cells were incubated with parthenolide (10  $\mu$ M) or curcumin (20  $\mu$ M) for 24h and the proliferation rate was estimated by measuring the number of viable cells (MTT test). §  $p < 0.05$  vs V403 cells, \*  $p < 0.05$  vs AS303 cells without inhibitor. B, V403 and AS303 cells were synchronized in 0.1% BSA for 48 h, parthenolide (10 $\mu$ M) was added during the last 24 hours. Cells were then harvested for subsequent analysis of the cell cycle. Means of four independent experiments are shown. §  $p < 0.05$  vs V403 cells, \*  $p < 0.05$  vs AS303 cells without parthenolide. C, Cyclin D1 expression was assessed by Western-blot analysis in protein extracts prepared from serum-starved V403 and AS303 cells treated or not with 10 $\mu$ M of the NF- $\kappa$ B inhibitor parthenolide for 24h using a specific anti-cyclin D1 antibody and the anti  $\alpha$ -tubulin antibody as equal loading control. Quantifications of cyclinD1/loading control ratio of 3 independent experiments are shown. §  $p < 0.05$  vs V403 cells, #  $p < 0.05$  vs AS303 cells without parthenolide.

Fig.4. Active NF- $\kappa$ B subunit p65 increases in AS303 cells.

A, V403 and AS303 cells were incubated with parthenolide (10 $\mu$ M) for 24h. DNA binding activity of the p65 subunit contained in nuclear extracts was measured using an NF- $\kappa$ B p65 ELISA-based transcription factor assay kit (TransAM assay). Results are expressed as optical density (OD) 450nm/655nm ratio. \*  $p < 0.05$  vs V403 cells without parthenolide, §  $p < 0.05$  vs V403 cells without parthenolide, #  $p < 0.05$  vs AS303 cells without parthenolide. B, Nuclear or total extracts prepared from cells treated or not with parthenolide (10 $\mu$ M) for 24h were submitted to Western-blot analysis using a specific anti-p65 antibody and the anti  $\alpha$ -tubulin antibody as equal loading control. Quantifications of nuclear p65/loading control ratio of 5 independent experiments are shown. §  $p < 0.05$  vs V403 cells, #  $p < 0.05$  vs AS303 cells without parthenolide.



Fig.5. Representative immunostaining for p65 protein in V403 and AS303 cells and in AS303-subcutaneous tumor. A and B: cells grown in chamber slides were immunostained with a specific phospho-NF- $\kappa$ B p65 antibody: immunoreactivity for phospho-p65 was absent in V403 cells (A), whereas several AS303 cells showed a strong nuclear immunostaining. C: sections from an AS303-subcutaneous tumor were immunostained with an anti-NF- $\kappa$ B p65 antibody. Most of the tumor cells showed a moderate cytoplasmic positivity for p65 and a strong nuclear immunopositivity was seen in many tumor cells, indicative of p65 phosphorylation and nuclear translocation. Photomicrographs are shown at 40x magnification.

Fig.6. A schematic model showing the regulatory role of menin in cyclin D1 expression. In normally expressing menin V403 cells, p65 and p50 translocate into the nucleus where they interact with menin, leading to a regulated transcriptional activity of the cyclin D1 gene. In low menin expressing AS303 cells, the amount of active p65 and p50 proteins increases in the nucleus, due to the weak expression of menin, leading to an upregulation of the transcriptional activity of the cyclin D1 gene.

Figure 1

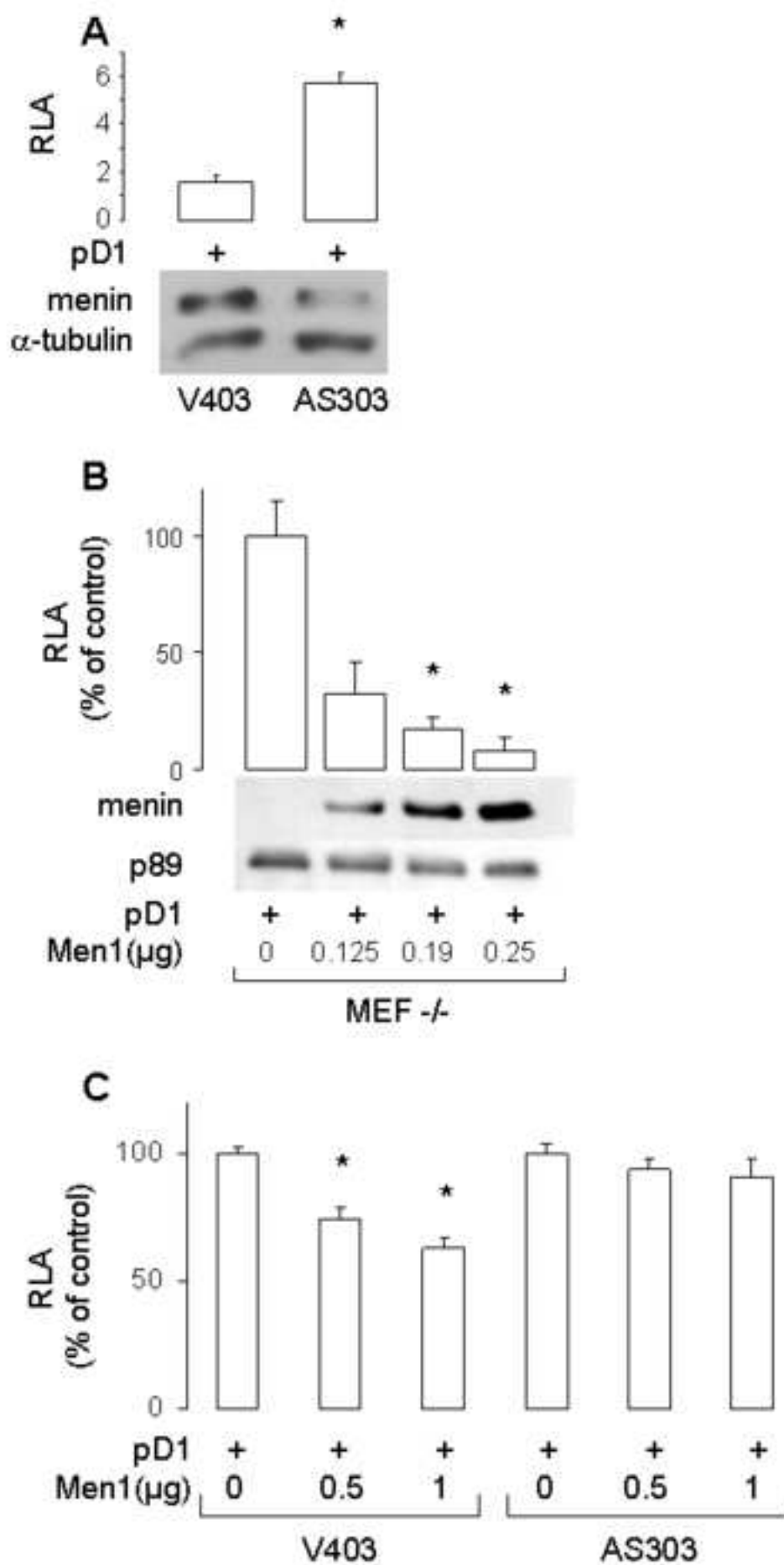


Figure 2

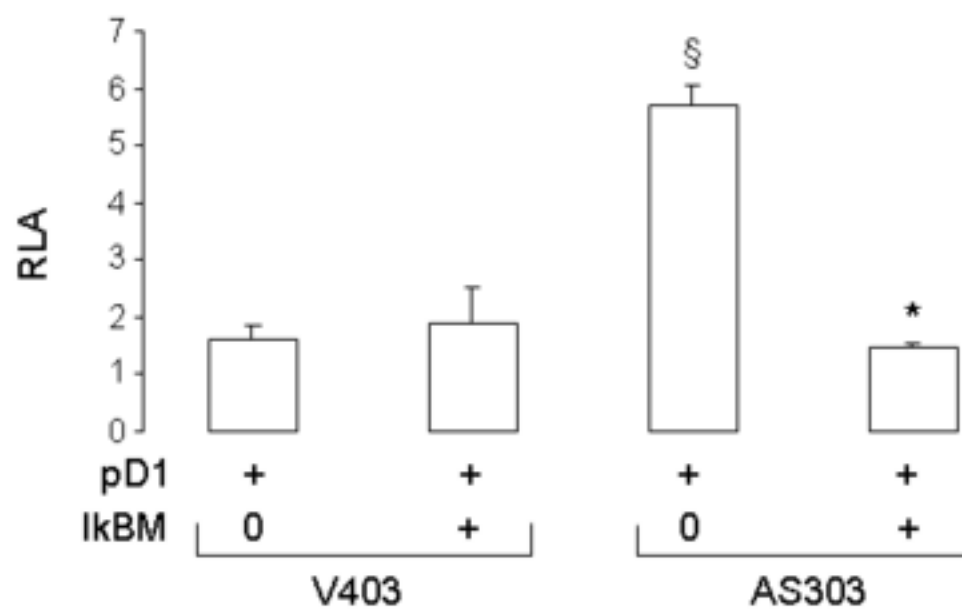


Figure 3

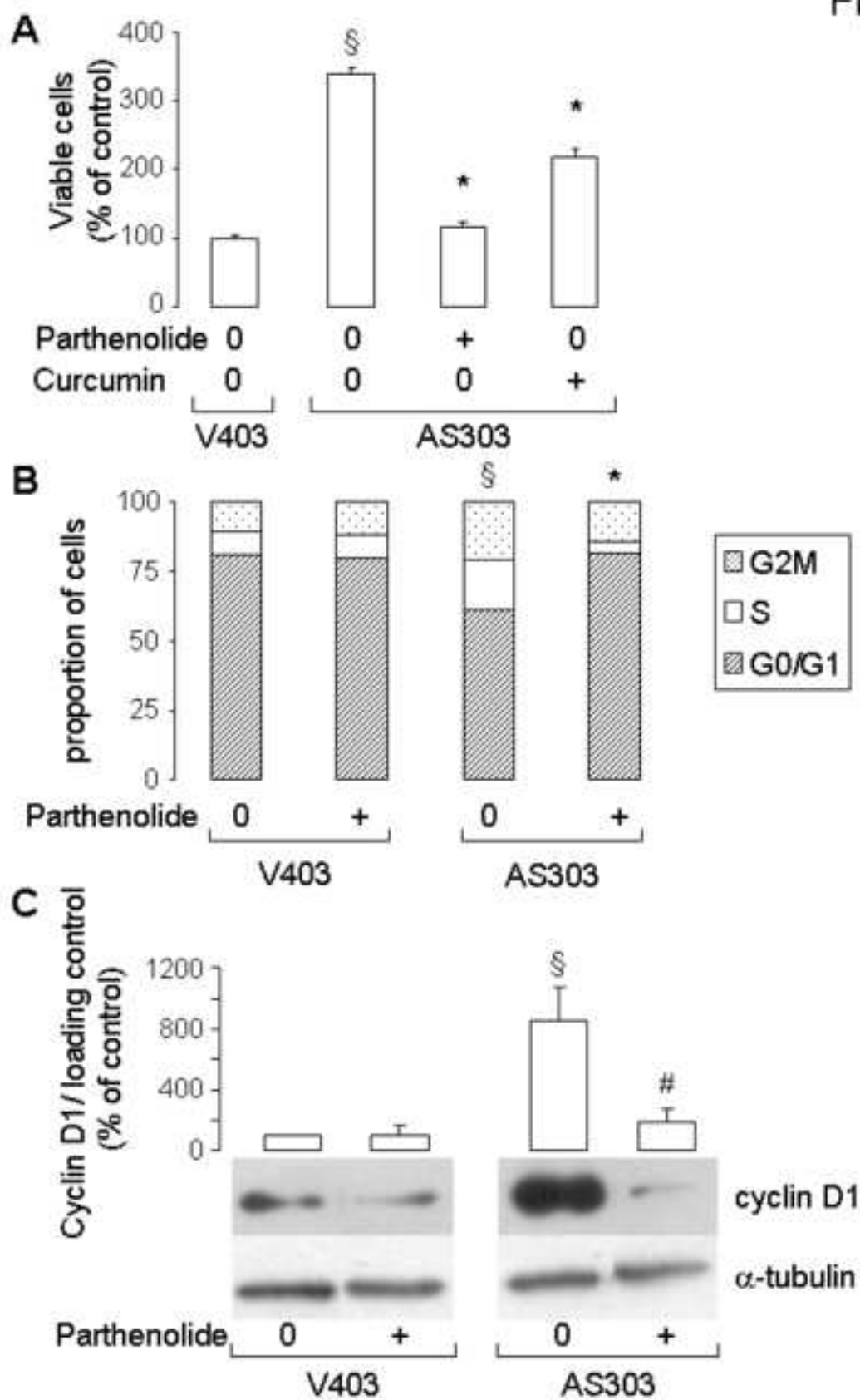


Figure 4

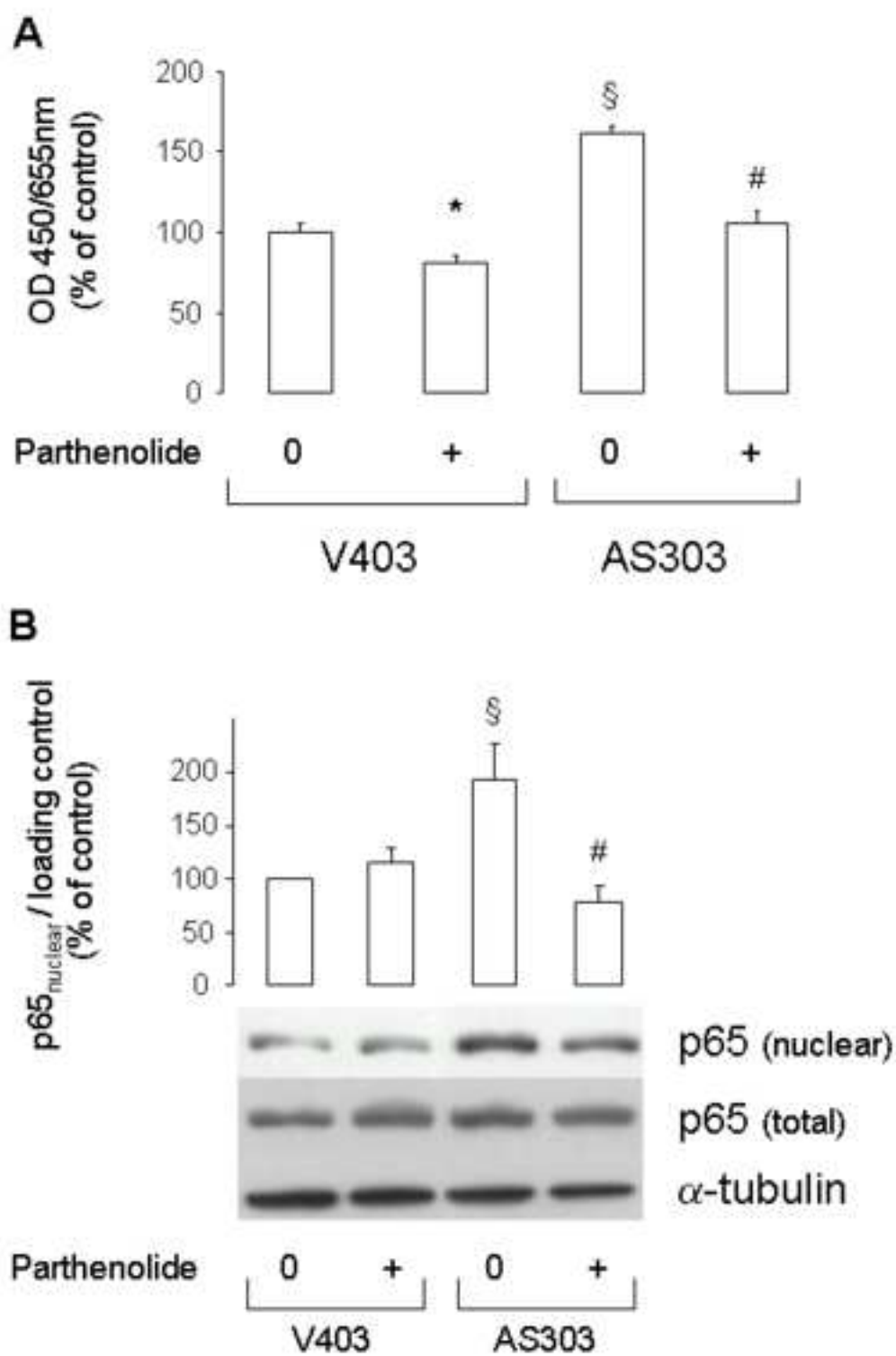


Figure 5

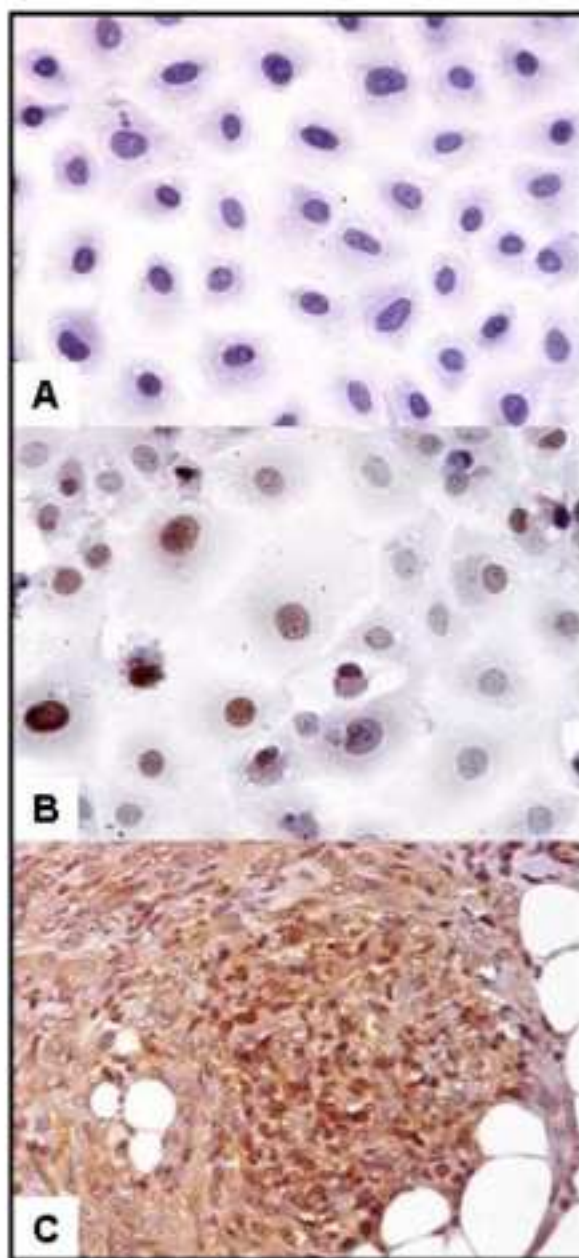


Figure 6

