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Upregulation of MMP-9 in MDCK epithelial cell line in response to expression of the Snail transcription factor

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Summary

Overexpression of the transcription factor Snail in epithelial MDCK cells promotes the epithelial-mesenchymal transition (EMT) and the acquisition of an invasive phenotype. We report here that the expression of Snail is associated with an increase in the promoter activity and expression of the matrix metalloproteinase MMP-9. The effect of Snail silencing on MMP-9 expression corroborates this finding. Induced transcription of MMP-9 by Snail is driven by a mechanism dependent on the MAPK and phosphoinositide 3-kinase (PI3K) signalling pathways. Although other regions of the promoter were required for a complete stimulation by Snail, a minimal fragment (nucleotides –97 to +114) produces a response following an increased phosphorylation of Sp-1 and either Sp-1 or Ets-1 binding to the GC-box elements contained in this region. The expression of a dominant negative form of MEK decreased these complexes. A moderate increase in the binding of the nuclear factor κB (NFκB) to the upstream region (nucleotide –562) of the MMP-9 promoter was also observed in Snail-expressing cells. Interestingly, oncogenic H-*Ras* (*Ras*V12) synergistically co-operates with Snail in the induction of MMP-9 transcription and expression. Altogether, these results indicate that MMP-9 transcription is activated in response to Snail expression and that it might explain, at least in part, the invasive properties of the Snail-expressing cells.

Key words: Snail, MMP-9, MAPK

Introduction

The Snail family of transcription factors can induce the process of cellular invasion and indeed, Snail itself regulates epithelial-mesenchymal transition (EMT) both during embryonic development and in epithelial cell lines (Batlle et al., 2000; Bolos et al., 2003; Cano et al., 2000). The phenotypic changes associated with this transition include both an increase in cell motility (Cano et al., 2000; Janda et al., 2002; Thiery, 2002) and in the production of extracellular matrix (ECM)-degrading enzymes, and they are always accompanied by the disruption of E-cadherin-mediated cell-cell adhesion (Christofori and Semb, 1999; Takeichi, 1995).

The matrix metalloproteinases (MMPs) is one of the most extensive families of ECM-degrading enzymes involved in EMT (Chakraborti et al., 2003; Kerkela and Saarialho-Kere, 2003). Along with other family members, matrix metalloproteinase-9 (MMP-9) plays a key role in cell migration and cell invasion both in physiological and pathological process (Freije et al., 2003; Fridman et al., 2003; Himelstein et al., 1994). Secretion of MMP-9 can be stimulated by a variety of factors including cytokines, growth factors, phorbol esters and bacterial endotoxins. Indeed, epidermal growth factor and amphiregulin stimulate the production of MMP-9 in the SKBr3 human breast cancer cell line (Kondapaka et al., 1997), as does heregulin-β1 (Yao et al., 2001). Moreover, TGF-β also activates MMP-9 in breast and prostate cancer cells (Samuel et al., 1992; Sehgal and Thompson, 1999; Welch et al., 1990) and in keratinocytes (Caulin et al., 1995). Significantly, recent studies in MDCK cells have shown that Snail mediates the EMT and the depletion of E-cadherin, triggered by TGF-β (Peinado et al., 2003). In addition, we have demonstrated that the loss of E-cadherin expression in mouse keratinocytes is related to an increase of MMP-9 mRNA levels and promoter activity (Llorens et al., 1998). However, oncogenic *Ras* and v-*Src* also induce MMP-9 expression in cancer cells and in the rat embryo (Ballin et al., 1988; Sato et al., 1993), suggesting that multiple cellular signalling pathways may drive MMP-9 expression (Gum et al., 1996).

One of the main mechanism through which extracellular signals are transmitted to the nucleus involves the activation of kinases related to the mitogen-activated protein kinase (MAPK) superfamily. Interestingly, these kinases have been shown to be responsible for the activation of MMP-9 expression in keratinocytes (McCawley et al., 1999; Zeigler et al., 1999), glioma tumour cells (Lakka et al., 2002) prostate cancer (Mehta et al., 2003) and melanoma cells (Govindarajan et al., 2003). In addition, phosphatidylinositol 3-OH kinase (PI3K) has been implicated in the EGF-induced cell surface association of pro-MMP-9 in ovarian cancer cells (Ellerbroek et al., 2001).

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Many studies of invasive tumours suggest that Snail and MMP-9 expression might be related since they have both been implicated in similar invasive processes (Blanco et al., 2002; Himelstein et al., 1994). However, an association between the expression of these two molecules has not been examined previously. To gain further insight into the mechanisms by which Snail induces the invasive phenotype, and whether or not MMP-9 is involved, we have analysed the molecular events underlying the EMT in the prototypic epithelial MDCK cell line. In this study, we demonstrate that Snail induces MMP-9 secretion via multiple signalling pathways that, in co-operation with oncogenic H-*Ras* (*Ras*V12), lead to the transcriptional upregulation of MMP-9.

Materials and Methods

Cell culture

MDCK-CMV and MDCK-Snail cells (Cano et al., 2000) were grown in DMEM-Ham's F-12 medium (Gibco, Grand Island, NY, USA) supplemented with 10% fetal bovine serum, sodium pyruvate, non-essential amino acids and L-glutamine (Gibco). The cultures were plated on plastic and incubated in 5% CO_2-95% air at 37°C in a humidified incubator. The cell lines were shown to be free of mycoplasma before performing in vitro experiments (assayed using the Mycoplasma Detection Kit for conventional PCR Venor GeM, purchased from Minerva Biolabs, Berlin, Germany).

Gelatin zymography

Exponential cultures of MDCK-CMV, MDCK-Snail and MDCK-Snail-siSnail cells were incubated for 24 hours with serum-free media. These conditioned media were centrifuged to clarify debris and concentrated by centrifugation in Centricon-30 microconcentrators (Amicon Inc., Beverly, MA, USA) prior to their use for gelatinase assays. Volumes for each cell line were adjusted to 12 μg protein as determined by BCA (Protein Assay Reagent, Pierce, Rockford, IL, USA). Conditioned medium from MXT-c1.1 cells (a metastatic mouse mammary carcinoma cell line) was included as a control for secreted gelatinases (Llorens et al., 1997).

Membrane-associated protein fractions were prepared from cultured cells by incubation for 30 minutes at 4°C with 200 μl of 2.5% Triton X-114 in cold TBS buffer and then centrifuged (3000 ***g*** for 15 minutes at 4°C) to remove Triton X-114-insoluble material. Homogenates were subjected to phase separation by incubation at 37°C for 5 minutes and spun at 3,000 ***g***. Aliquots of the hydrophobic phase containing integral membrane protein were analysed by zymography.

Gelatin zymography was performed as described elsewhere (Llorens et al., 1998). Samples were mixed with SDS sample buffer without reducing agent and separated on 7.5% SDS-PAGE gels containing 0.1% gelatin. The gels were incubated at 37°C after removing the SDS by washing with 2.5% Triton X-100-containing buffer. The gels were stained with Coomassie Brilliant Blue R250, and the gelatinolytic activities were detected as clear bands against a blue background.

RT-PCR and primers

For RT-PCR, 100 ng of Poly(A)$^+$ RNA samples were reverse transcribed in a total reaction volume of 10 μl containing: 1× RT buffer (50 mM Tris-HCl pH 8.3, 75 mM KCl and 3 mM $MgCl_2$), 20 U RNase inhibitor (Promega Madison, WI, USA), 10 mM dithiothreitol, 50 mM deoxyribonucleoside triphosphates (dNTPs), 0.1 mM random primer (Perkin Elmer), and 200 U Moloney murine leukemia virus reverse transcriptase (M-MLV-RT; Life Technologies, Gaitherburg, MD, USA). The mixture was incubated at 37°C for 60 minutes and 95°C for 5 minutes, using a programmable thermal cycler (PTC-100 TM; MJ Research, Watertown, MA, USA). The PCR reaction was performed with the specific forward and reverse primers for canine MMP-9 described in GenBank, accession number: AF169244 (MMP-9 forward (F): 5′ GGTCTGGGTGACTCCAAAG-CC 3′ and MMP-9 reverse (R): 5′ GGTGAGGGTAGTGGTGTGTCT 3′) in a thermal cycler over 35 high-stringency cycles (denaturation at 94°C for 30 seconds, annealing at 65°C for 1 minute and extension at 72°C for 1 minute). Oligodeoxynucleotides were purchased from Pharmacia Biotech (Genosys, Cambridge, UK). Cyclophillin-specific primers (CYCLO F: 5′ CACCACATGCTTGCCATCC 3′ and CYCLO R: 5′ CTCCTTTGAGCTGTTTGCAG 3′). Specific primers for mouse Snail and canine E-cadherin or GAPDH were used in parallel reactions proceeding as described elsewhere (Bolos et al., 2003; Peinado et al., 2003). The PCR products were visualised in ethidium bromide-stained agarose gels and the identity of the PCR products was confirmed by automated DNA sequencing at the IRO DNA Sequencing Core Facility after cloning into the pCR2.1 vector (Invitrogen, Inc., Carlsbad, CA, USA). All the PCR reactions were performed at least twice and the results were similar in all cases.

Western blot analysis

SDS-containing polyacrylamide gel electrophoresis (SDS-PAGE) was carried out as described previously, and proteins separated on the gel were transferred to a PVDF membrane. Following blocking with 5% fat-free dry milk in Tris-buffered saline, the membrane was probed with primary antibodies specific to MMP-9 protein (Rodriguez-Manzaneque et al., 2001) (Chemicon International Inc., Temecula, CA, USA; cat. no. AB 19047) at a dilution of 1:1000; specific to total Erk1 and Erk2, the p44/42 MAP kinase polyclonal antibody (Cell Signaling Technology, Beverly, MA, USA) was used at a 1:1000 dilution; and the anti-Sp-1 monoclonal antibody (clone 1C6, BD Pharmingen Technical, BD, San Diego, CA, USA) at 1:200 dilution. For detection of the levels of Akt phosphorylation we used the PhosphoPlus Akt (Ser 473) antibody (New England BioLabs, Beverly, MA, USA) at 1:200 dilution. The membranes were further probed with HRP-conjugated goat anti-rabbit or rabbit anti-mouse immunoglobulin (Bio-Rad Laboratories, Hemel Hempstead, UK) for 1 hour at room temperature (1:1000). Antibody binding was visualized by ECL (Amersham Biosciences, Upsala, Sweden).

MMP-9 immunostaining

Cells cultured on coverslips were fixed with 3% paraformaldehyde in PBS containing 75 mM lysine. After blocking with 5% goat serum and 3% bovine serum albumin in TBS for 1 hour at room temperature, cells were reacted with anti-MMP-9 rabbit polyclonal antibody (Chemicon Int. Inc., cat. no. AB 19047) (Rodriguez-Manzaneque et al., 2001) and incubations were carried out at room temperature for 1 hour using a dilution of 1:500 in Tris-buffered saline (TBS). $CaCl_2$ (1 mM) was included throughout the washing and incubation for the staining with the anti-MMP-9 antibody. Fluorescein-conjugated goat anti-rabbit IgG was used to visualize the antigen signal. DAPI was used to stain the nuclei. Negative controls were performed by incubation of fixed cells in the absence of primary antibody.

MMP-9 promoter constructions

The mouse wild-type MMP-9 promoter sequences [nucleotides (nts) –1170 to +142] was amplified by PCR from genomic DNA using specific oligonucleotides (pMMP9-1170F*:* 5′ TGGGAGAACACC-CAGCTCTCTC 3′ and pMMP9R: 5′ CTCTGCCAGCTGGTGTCCG 3′). The amplified fragment was cloned into the pCR2.1 vector (Invitrogen, Inc., Carlsbad, CA, USA) and then into the *Kpn*I/*Xho*I sites of pGL2-basic vector (Promega Corp.) fused to a luciferase

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reporter gene. Several fragments of the 5′ end of the mouse MMP-9 promoter were generated by PCR as follows: for the pMMP9-588 construct (nts –588 to +142) the primers (p MMP9-558F*:* 5′ GGAGCTAGGGGTTTGCCC 3′ and pMMP9R: 5′ CTCTGCCA-GCTGGTGTCCG 3′) were used, and the pMMP9-389 construct was generated by amplifying of region spanning from nts –389 to +142 with the forward and reverse primers (pMMP9-389 F: 5′ GGT-CTCGGGCCTCAGGTCTC 3′ and pMMP9 R: 5′ CTCTGCCAG-CTGGTGTCCG 3′). For the pMMP9-117 construct (nts –117 to +142) the primers (pMMP9-117F: 5′ ACACACACGCTGAGTCAG-GCATA 3′ and pMMP9R: 5′ CTCTGCCAGCTGGTGTCCG 3′) were used; for the pMMP9-97 construct (nts –97 to +142), pMMP9-97F: 5′ TAAGCCTGGAGGGGAGGGGCGG 3′ and pMMP9R: 5´ CTC-TGCCAGCTGGTGTCCG 3´; and finally for pMMP-75 construct (nts –75 to +142) the primers pMMP9-75 F: 5′ GGTCACTGATTCCGT-TTTA 3′ and pMMP9R: 5′ CTCTGCCAGCTGGTGTCCG 3′. The PCR reaction with *Taq* polymerase and specific primers for the MMP-9 promoter constructs was performed in a thermal cycler over 30 high-stringency cycles (denaturation at 94°C for 30 seconds, annealing at 55 °C for 1 minute and extension at 72 °C for 1 minute). The PCR products were cloned into the pCR2.1 vector and then into the appropriate site of pGL2-basic vector directing luciferase reporter expression. The constructs containing the luciferase gene driven by the specific promoter regions were called pMMP9-1170luc (construct I); pMMP9-588luc (construct II); pMMP9-389luc (construct III); pMMP9-117luc (construct IV); pMMP9-97luc (construct V) and pMMP9-75luc (construct VI) according the regions described above.

Site-directed mutagenesis was used to introduce point mutations into the putative binding sites in the MMP-9 promoter. The PCR reactions for mutagenesis were performed on 10 ng of wild-type construct V as a template and using 100 ng of each mutated primer set, 25 mM of each dNTP, 2.5 U of pFu-Turbo-DNA polymerase (Stratagene) and its corresponding buffer. After PCR, the parental supercoiled double-stranded DNA was digested with *Dpn* I (Roche Diagnostics, Mannheim, Germany) and competent *E. coli* cells (TOP F10) were transformed by thermal shock. Mutant construction at nts –89 and –87 was generated by using the forward primer pMMP9-M$^{89\text{-}87}$F: 5′ TAAGCCTG**a**A**t**GGGAGGGGCGG 3′. Mutant construction at nts –85 and –82 was generated by using the forward primer pMMP9-M$^{85\text{-}82}$F:5′ TAAGCCTGGAGG**t**GA**t**GGGCGG 3′. The promoter constructs and the novel mutations were confirmed by sequencing.

Expression vectors and transfections

pcDNA3-DN-MEK1, a dominant negative expression vector of MEK1 was provided by Ana Aranda (Instituto de Investigaciones Biomédicas, CSIC, Madrid, Spain); pCELF- H-*Ras* (RasV12) and pCELF-N-*Ras* (RasE12) were a gift from Piero Crespo (Instituto de Investigaciones Biomédicas, CSIC, Madrid, Spain). The amount of DNA transfected was kept constant by addition of the appropriate amount of empty vector.

As indicated in the figures, the cells were incubated for 5 hours with 50 μM of PD 098059 (or UO126); 40 nM wortmannin and 10 μM SB 203580 purchased from Sigma.

For generation of siRNA expression vectors, oligonucleotides containing specific sequences against mouse Snail nucleotide 19 (5′-GATGCACATCCGAAGCCAC-3′) were cloned into the pSuperior-Puro vector (Oligoengine, Seattle, WA), according to manufacturing instructions. pSuperior-Puro and pSuperior-siSnail vectors were transfected in the indicated cell lines using lipofectamine (Gibco-BRL). Stable transfectants were generated after selection with 1 μg/ml puromycin (siRNA-derived cells) for 2-4 weeks and characterized for expression of Snail and E-cadherin.

Transient transfections were carried out with lipofectamine plus reagent (Promega Corp.), essentially following the manufacturer's instructions. MDCK-CMV cells and MDCK-Snail stable transfectants (MDCK-Snail) were transiently cotransfected with 1 μg of the MMP-9 promoter of wt and deletion mutants fused to a luciferase reporter gene and 100 ng of TK renilla plasmid in 6-well plates. Cells were transfected in FBS-free medium for 5 hours and switched to the media with 10% FCS for an additional 24 hours and then collected to determine the luciferase/renilla activity. The cells were lysed in 80 μl of 1× lysis buffer (Promega Corp.), and after 3 freeze/thawing cycles and centrifuging at 3000 ***g*** for 10 minutes. Luciferase and renilla activities were measured in supernatants using the Dual-Luciferase Reporter Assay kit (Promega Corp.). As indicated, MDCK-CMV cells were cotransfected with the indicated expression vectors in the same conditions as described above.

Electrophoretic mobility shift assays

For electrophoretic mobility shift assays (EMSA), 10 μg of each forward and reverse oligonucleotides (synthesized by Life Tech., Invitrogen) were annealed in a final reaction volume of 20 μl with 1× buffer M (Boehringer Mannheim). After heating for 5 minutes, the temperature was gradually lowered overnight. A double-strand oligonucleotide (100 ng) was radiolabelled using T4 polynucleotide kinase (Promega) and [γ-^{32}P]ATP (Amersham Pharmacia Biotech) according to the manufacturer's instructions. The radiolabelled oligonucleotide was purified on SC-200 columns (Pharmacia) and used as a probe. Mobility shift assays were performed with 10 μg MDCK-CMV and MDCK-Snail nuclear extracts. Nuclear protein extraction was carried out by the procedure described previously (Perez-Moreno et al., 2001) and mixed with 1×10^5 c.p.m. of radiolabelled probe. The different probes used were: NFκB binding site wild type (NFκB wt: 5′ TGCCCCATGGAATTCCCCAA 3′) at nucleotide (nt) –562 and its mutant (NFκB m: 5′ TGCCCCATGGAATTCCCCAA 3′); E-box putative binding site wild-type sequence from nts –650 to –630 of MMP-9 promoter (E-box wt: 5′ TGCTCCCACATGTGTGTGTC 3′); E-pal E-cadherin promoter (E-pal wt: 5′ GGCTGCCACCTGCAGGTGCGTCCC 3′); Sp-1 binding site wild-type sequence from nts –97 to –76 of the MMP-9 promoter that contains a putative low affinity Sp-1 binding site, a high affinity Sp-1 binding site and Ets-1 binding sites (Sp-1 wt: 5′ TAAGCCTGGAGGGGAGGGCGG 3′) and the wild-type sequence from nts –85 to –65 (5′ GGA GGGGCGGGGTCACTGAT 3′) that contains a putative high affinity Sp-1 and an Ets-1 binding sites. For super-shift experiments 1 μg of the corresponding polyclonal antibody: anti Sp-1 (sc-59x); anti NFκB p65 (sc-109) and αnti-Ets-1 (sc-111x) or non-immune rabbit IgG (all from Santa Cruz Biotechnology, Santa Cruz, CA) was added to the reaction mixture and incubated for 20 minutes on ice. Where indicated, 5 μg of rabbit polyclonal anti-Snail antibody (Bolos et al., 2003) was added and incubated before addition of the labelled probe. In competition assays, 500-fold molar excess of the corresponding cold double-stranded DNA oligonucleotide was added and the reaction was incubated for 10 minutes on ice. DNA-protein complexes were separated on 6% non-denaturing polyacrylamide gels in 0.5× TBE (Tris-borate-EDTA) at room temperature. The gels were dried and the complexes were visualised by autoradiography.

Results

Snail induces MMP-9 secretion in MDCK cells

We previously demonstrated that the expression of Snail in the prototypic epithelial MDCK cell line triggers phenotypic changes commonly known as EMT (Cano et al., 2000). Snail-induced disruption of cell-cell contacts, cell dissociation and an increase in cell motility, which is reminiscent of the behaviour of MMP-9 expressing cells at the invasive edges of tumours (Kupferman et al., 2000). To examine whether MMP-9 was involved in this process, MDCK cells that ectopically

expressed mouse Snail were analysed (Cano et al., 2000). A significant increase in MMP-9 gelatinolytic activity was found in these cells, but not of another closely related gelatinase, MMP-2. In zymographic assays, MMP-9 activity was detected both in the extracellular media and associated with the cell membrane (Fig. 1A). The predominant band was detected at 110 kDa corresponding to the proactive form that becomes active during zymography. RT-PCR analysis indicated that MCDK-Snail cells contain more MMP-9 mRNA than MDCK-CMV cells (Fig. 1B), and the increase in MMP-9 expression was clearly observed by immunofluorescence at the surface of MDCK-Snail cells, with a mixed granular and fibrillar pattern (Fig. 1C). Western blot analysis using the same specific antibody also indicated the increased level of MMP-9 protein, detected as two bands at 110 kDa and 90 kDa, both recognized by the antibody (Fig. 1D), although the correspondence of the 90 kDa band with the active form could not be definitively established.

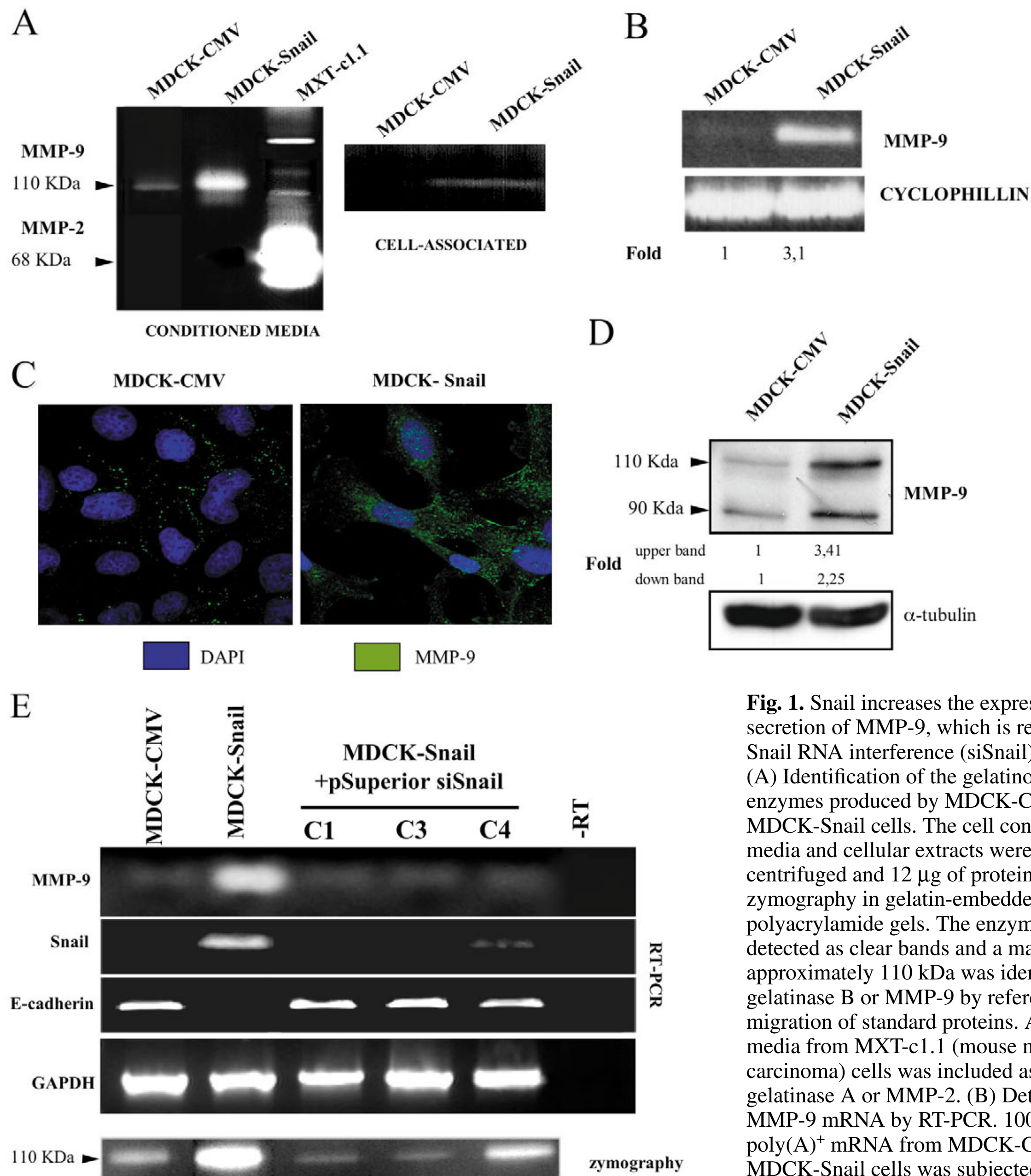

Fig. 1. Snail increases the expression and secretion of MMP-9, which is relieved by Snail RNA interference (siSnail). (A) Identification of the gelatinolytic enzymes produced by MDCK-CMV and MDCK-Snail cells. The cell conditioned media and cellular extracts were collected, centrifuged and 12 μg of protein analysed by zymography in gelatin-embedded SDS polyacrylamide gels. The enzymes were detected as clear bands and a major band of approximately 110 kDa was identified as gelatinase B or MMP-9 by reference to the migration of standard proteins. A conditioned media from MXT-c1.1 (mouse mammary carcinoma) cells was included as control for gelatinase A or MMP-2. (B) Detection of MMP-9 mRNA by RT-PCR. 100 ng of poly(A)$^+$ mRNA from MDCK-CMV and MDCK-Snail cells was subjected to RT-PCR. The amplification of cyclophillin was used to normalise for loading (lower panel). (C) Immunofluorescence detection of MMP-9 in MCDK-CMV (left) and MDCK-Snail (right) cells. Note the intense fibrillar and granular staining in MDCK-Snail cells. (D) Cells collected from subconfluent cultures of MDCK-CMV and MDCK-Snail cells were lysed, and the expression of MMP-9 was analysed by western blotting using an anti-MMP-9 polyclonal antibody. α-Tubulin was used as loading control. (E) Snail silencing blocks the induction of MMP-9 expression. Upper four panels: RT-PCR analysis of MMP-9, Snail and E-cadherin mRNA levels in MDCK-CMV, MDCK-Snail and in three independent stable clones (C1, C3 and C4) generated after siSnail transfection (MDCK-siSnail) in MDCK-Snail cells. GAPDH mRNA levels are shown as loading control. Lower panel: the effects of Snail interference on secreted MMP-9 were analysed by zymography in gelatin-embedded SDS polyacrylamide gels.

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To provide a link between Snail expression and MMP-9 induction we used a Snail interference technique with a 19-mer siRNA oligonucleotide directed to the N-terminal region of the first zing finger of mouse *Snail* mRNA (position 573-591 in mouse cDNA; accession number NM011247). To this end, the pSuperior-siSnail vector was stably transfected in the MDCK-Snail cells and its effect on E-cadherin and MMP-9 expression was analysed by semiquantitative RT-PCR analysis. The efficiency of Snail interference was observed in three independent clones with either complete absence or very low levels (less that 10% over the corresponding control in clone C4) of Snail mRNA (Fig. 1E). Analysis of E-cadherin mRNA levels indicated robust expression of E-cadherin in all independent clones with silenced Snail expression. Interestingly, the stable interference of Snail obtained in the MDCK-Snail system, apart from reactivating E-cadherin expression, also abrogated the MMP-9 mRNA in all analysed independent clones, while control MDCK-Snail cells showed robust levels of MMP-9 transcript. In agreement with these data, an important reduction of secreted MMP-9 activity after Snail interference in the individual clones as compared to MDCK-Snail cells was detected by zymography (Fig. 1E lower panel). Only the MDCK-Snail C4 clone shows a less effective silencing of Snail compared to the other analysed clones, since some level of Snail transcript is still detected (Fig. 1E, Snail panel). Indeed, MMP-9 activity could be detected in the conditioned medium from C4 clone (Fig. 1E, lower panel), even when no apparent MMP-9 mRNA is observed by RT-PCR (Fig. 1E, upper panel). A similar situation to the C4 clone is in fact observed in control MDCK-CMV cells (compare the corresponding lanes in Fig. 1E). Controls performed with a siRNA oligonucleotide directed to the unrelated gene EGFP showed no alteration in any of the analysed markers (Snail, E-cadherin and MMP-9) as compared with control MDCK-Snail cells (data not shown).

Snail increases the activity of the MMP-9 promoter in MDCK cells

To determine whether the increase in MMP-9 mRNA levels was due to increased transcription, we analysed the activity of the MMP-9 promoter using a luciferase reporter gene. The activity of a 1.17 kb fragment of the MMP-9 mouse promoter region was examined as well as several deletion mutant constructs (Fig. 2A). MDCK cells that

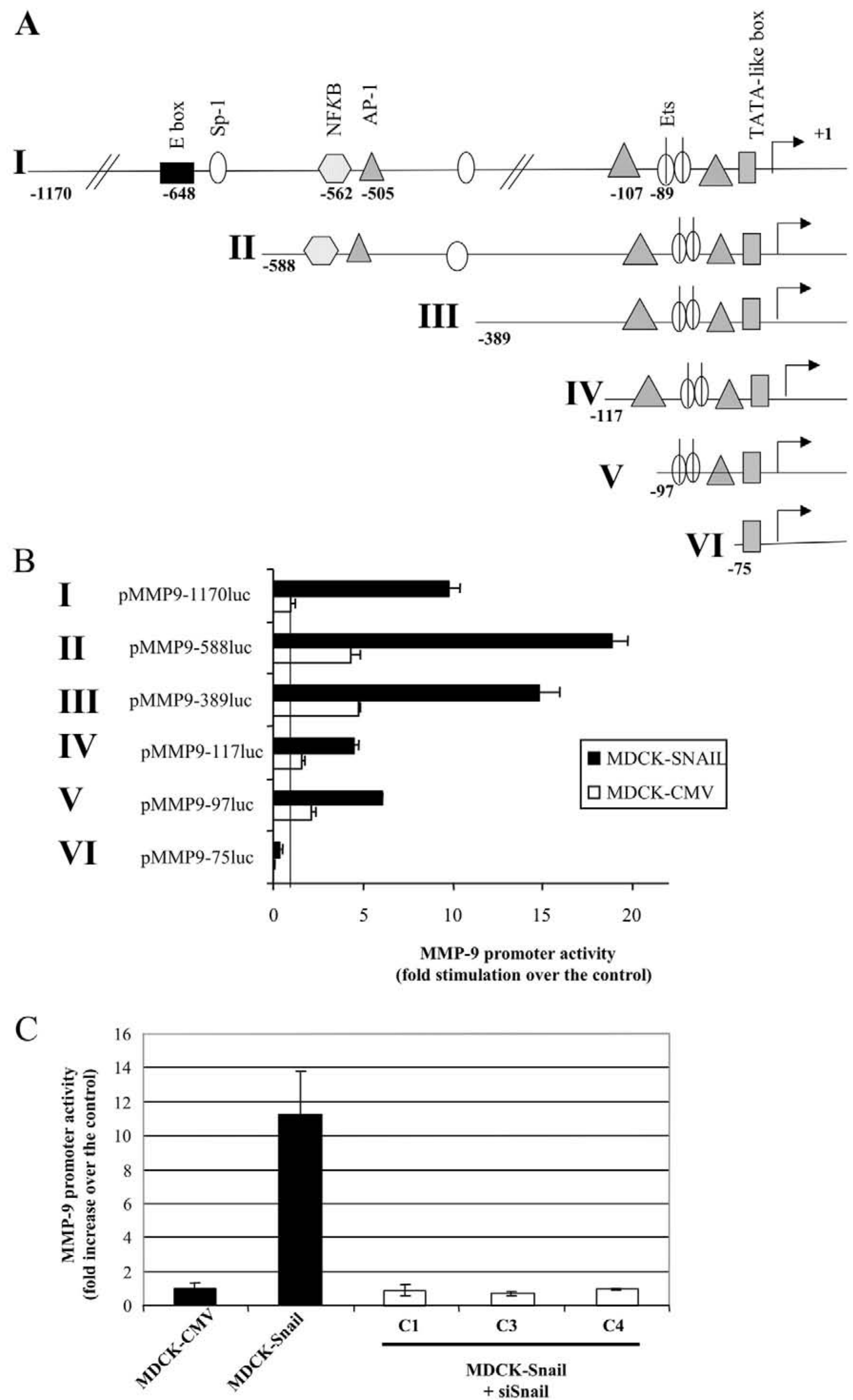

Fig. 2. Stable expression of Snail induces MMP-9 promoter activity and is relieved by siSnail. (A) Schematic representation of the MMP-9 promoter and the deletion mutant constructs (I-VI), indicating the position of potential regulatory control elements. (B) Diagram showing the increase in activity of the different constructs transiently expressed in MDCK-CMV cells (white bars) or MDCK-Snail cells (black bars). Luciferase and renilla activities of each promoter construct are relative to that obtained with the pGL2 control plasmid, expressed as the mean±s.d. of three independent experiments. (C) Induction of MMP-9 promoter mediated by Snail is relieved by siSnail. Analysis of –1170 MMP-9 promoter activity (construct I) in control (MDCK-CMV, MDCK-Snail) and in the indicated individual clones obtained after stable transfection of Snail siRNA. Promoter activity was determined as in B. Results represent the mean±s.d. of four independent experiments.

ectopically express Snail show an approximately 10-fold increase of luciferase activity when driven by the pMMP9-1.170luc promoter (construct I in Fig. 2B). In addition, the pMMP9-588luc construct (construct II) displayed a 4-fold higher level of activity in MDCK-Snail cells compared to MDCK-CMV control cells. The Snail-mediated induction of the shorter constructs IV and V was twofold, indicating that sequences present in the proximal promoter were contributing to the activation. (Fig. 2B, promoter constructs IV and V). In agreement with these results, analysis of MMP-9 promoter activity in MDCK-Snail clones in which efficient Snail blockade was obtained, showed a strong reduction of the transcriptional activity. As shown in Fig. 2C, the MMP-9 promoter activity analysed in the stable siSnail clones was similar to that of MDCK-CMV cells. Taken together, the promoter analysis data indicated a direct link between Snail expression and induction of MMP-9. To confirm this observation, the effect of transient Snail expression was analysed by co-transfecting the pMMP-9-reporter plasmids into MDCK cells with 1 μg of the full-length mouse Snail cDNA. As shown in Fig. 3A, luciferase activity was increased in MMP-9 reporters I to V, when Snail was co-expressed with these constructs in MDCK cells (Fig. 3A). Concomitantly to these experiments, transfection of the siSnail expression vector completely abrogated the induction of MMP-9 promoter activity by Snail (Fig. 3B).

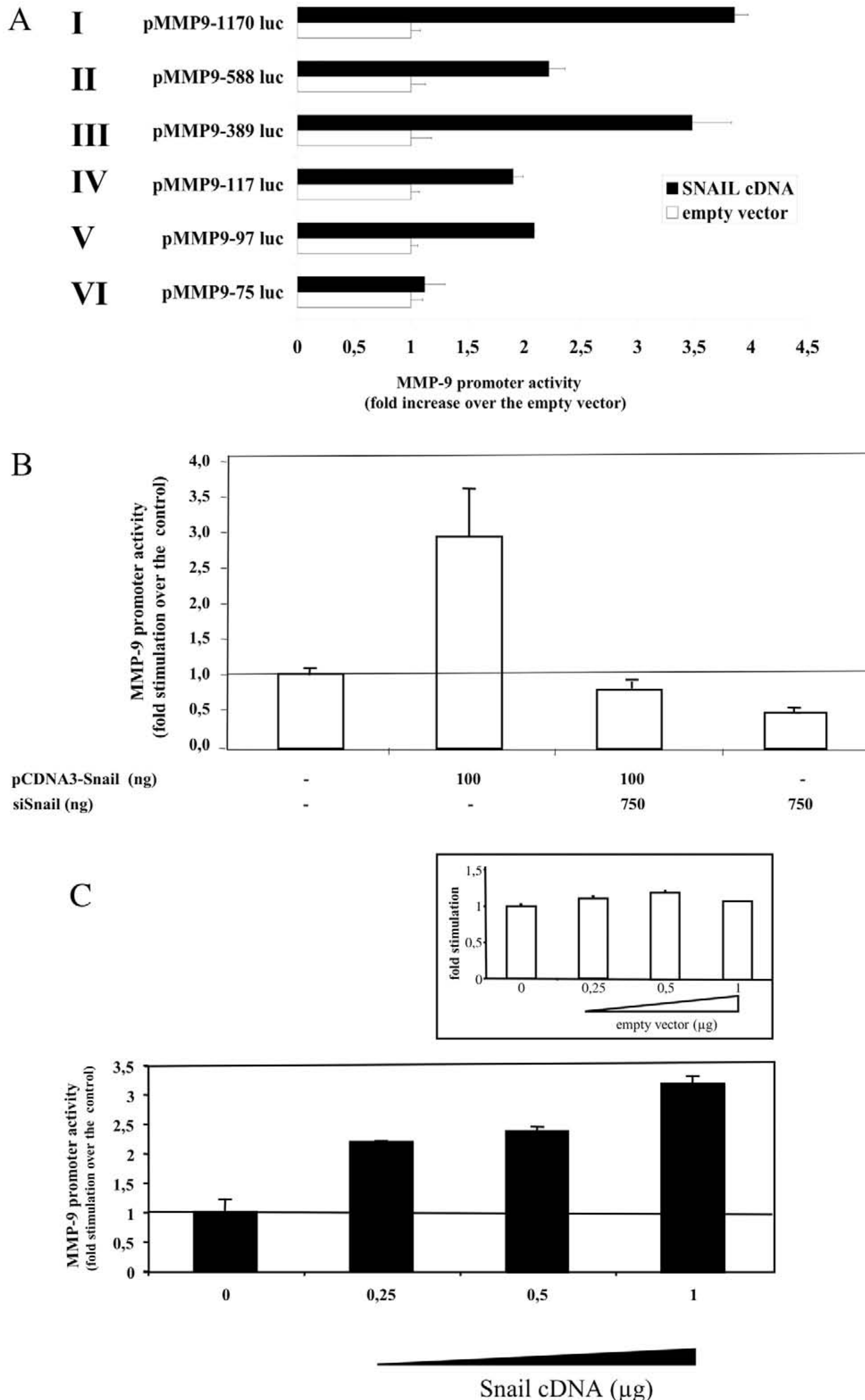

Fig. 3. Transient expression of Snail induces MMP-9 promoter activity and is relieved by siSnail. (A) Activity of the different constructs was analysed in MDCK-CMV cells transiently transfected with Snail cDNA (black bars) or with the empty expression vector (white bars). The effect of Snail is represented for each MMP-9 reporter as fold stimulation of the activity over the empty vector as the mean±s.d. of three independent experiments. (B) The effect of siSnail on MMP-9 promoter activity (construct I) was measured in MDCK-CMV cells growing in T24 plates, after transfection of the indicated amounts of the different vectors and compensated with the empty plasmid. Promoter activity is given as fold stimulation over that with the empty plasmid (mean±s.d. of four independent experiments). (C) Dose-dependent effect of Snail cDNA on the –389 MMP-9 (construct III) promoter activity was analysed in MDCK-CMV cells as described in A. The amount of the Snail cDNA was compensated with empty plasmid up to 1 μg of total DNA. Promoter activity is given as fold stimulation over that obtained in the absence of Snail cDNA. The small panel at the top shows the dose-dependent effect of the empty plasmid on the activity of this promoter construct. Values are the mean±s.d. of three independent experiments.

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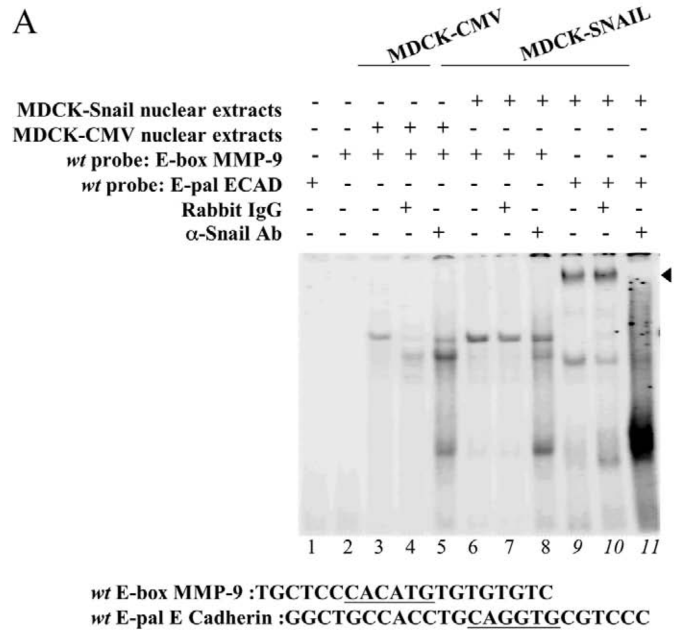
Binding analysis of transcription factors to the MMP-9 promoter in Snail expressing cells

Snail has previously been shown to bind to DNA elements (CAGGTG) that conforms to the E-box consensus (CANNTG). One E-box element is detected at position –648 in the –1.17 MMP-9 promoter (Fig. 2A), but is absent from the –588 MMP-9 promoter and other deletion constructs that also show increased promoter activity in the presence of Snail (Fig. 2B and Fig. 3A). To further explore the capacity of Snail to modulate the activity of the proximal MMP-9 promoter, the activity of the pMMP9-389luc construct (III) was analysed by transient transfection with increasing doses of Snail cDNA. Results shown in Fig. 3C demonstrated that Snail cDNA stimulated the activity of the MMP-9 promoter in a dose-responsive manner. Other deletion constructs of the MMP-9 promoter also responded to transient Snail expression with an approximately twofold increase in activity. These results suggest that additional regulatory elements in the MMP-9 gene are involved in Snail-mediated upregulation of the MMP-9 promoter.

To analyse the potential of Snail to interact with the MMP-9 promoter, band-shift studies (EMSA) were performed using the putative E-box element (CACATG) of the promoter. As a control, we included a labelled probe corresponding to the E-pal element of the mouse E-cadherin promoter containing two adjacent E-boxes (Behrens et al., 1991) containing a consensus Snail binding element. As can be observed in Fig. 4A, the nuclear extracts of MDCK-Snail cells did not bind the E-box element at position –648 of the MMP-9 promoter whereas the same extracts bound to the E-pal element (CAGGTG) of the E-cadherin promoter that has been shown to bind this factor with high affinity (Bolos et al., 2003). Indeed, a large complex (indicated by an arrowhead in Fig. 4A,B) was generated in MDCK-Snail cells by using the E-pal probe, which was effectively competed by an excess (×500) of the cold wild-type oligonucleotide but not competed by a similar excess of the cold mutant E-pal oligonucleotide (Fig. 4B). The specificity of the Snail complex was further confirmed by the use of the anti-Snail polyclonal antibody (shown in Fig. 4A, lane 11) and is in close agreement with previous results (Bolos et al., 2003). The rest of the complexes were considered nonspecific, or unrelated to Snail, since they were not recognized by the Snail antibody. Taken together, the results indicate that the activation of the MMP-9 promoter by Snail is not achieved by direct binding of this transcription factor to this nt –648 E-box element and that binding of other transcription factors should be responsible.

NFκB has been reported to regulate MMP-9 expression in different systems (Farina et al., 1999; Kondraganti et al., 2000). Therefore, we analysed whether extracts from stable MDCK-Snail cells could bind to the NFκB transcription factor motif (TGGAATTCCCCA), included in the construct II, in EMSA assays. Similar complexes (indicated as 'i and ii' in Fig. 5)



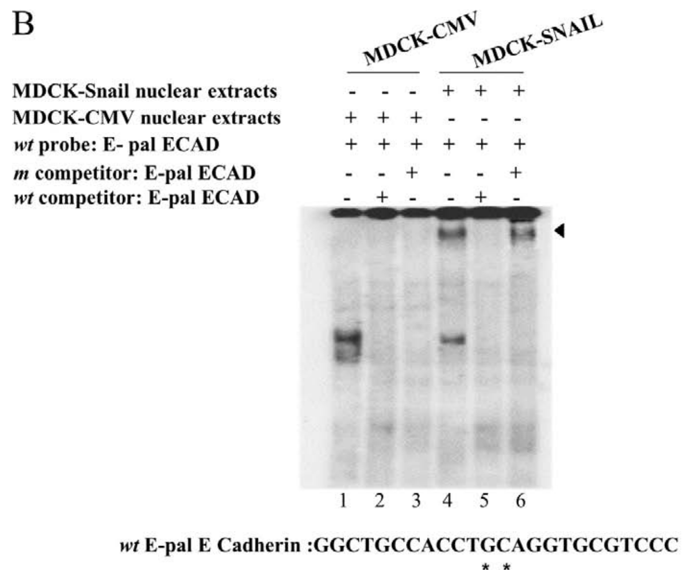


Fig. 4. Nuclear extracts from MDCK-Snail cells did not bind to the putative E-box element of the MMP-9 promoter but bound to the E-pal element of E-cadherin promoter. (A) Nuclear extracts from MDCK-CMV and MDCK-Snail cells were analysed in band-shift assays using the ^{32}P-labelled E-box wild-type probe of the MMP-9 promoter containing the putative E-box at nt –648 (lanes 2-8), or the E-pal element of the mouse E-cadherin promoter (lanes 1, 9-11). Lanes 1 and 2 show EMSA in which the nuclear extract was not added. The retarded complexes were detected when using the E-pal probe of the E-cadherin promoter and are indicated by a black arrowhead. Incubation of the MDCK-Snail nuclear extracts in the presence of control rabbit IgG or an anti-Snail antibody is shown in lanes 10 and 11, respectively. The complete sequence of the E-box MMP-9 probe and the E-pal E-cadherin probe are shown at the bottom of the figure with position of E-boxes underlined. The gel shown is representative of at least two independent experiments. (B) Nuclear extracts from MDCK-CMV and MDCK-Snail cells were analysed in band-shift assays using as a probe the E-pal element of the E-cadherin promoter. Nuclear extracts were incubated with the ^{32}P-labelled E-pal probe in the presence of 500-fold molar excess of wild-type (lanes 2 and 5) or mutant cold oligonucleotides (lanes 3 and 6). A black arrowhead indicates a retarded complex. The complete sequence of the E-pal probe is shown at the bottom with the position of the mutated nucleotides indicated by asterisks.

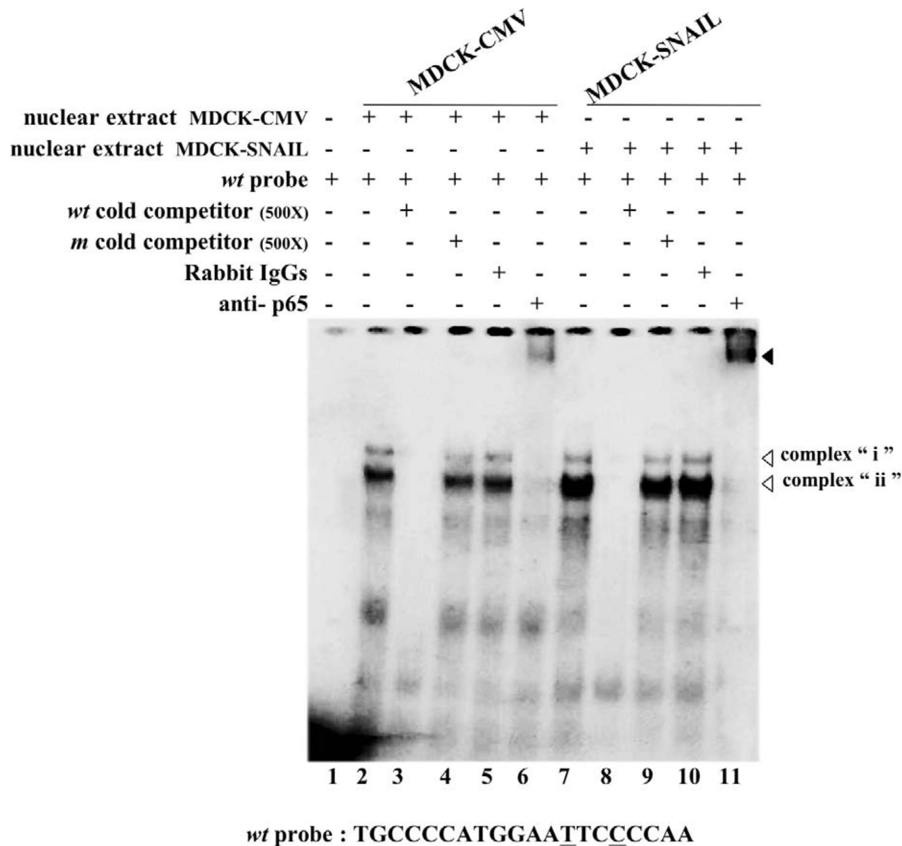


Fig. 5. Nuclear extracts from MDCK-Snail cells contain specific NFκB-binding complexes. Nuclear extracts from MDCK-CMV and MDCK-Snail cells were analysed in band-shift assays showing the binding of nuclear proteins to the ^{32}P-labeled NFκB wild-type probe, in the absence or presence of 500-fold molar excess of wild-type (lanes 3 and 8) or mutant cold oligonucleotides (lanes 4 and 9) or in the presence of anti-p65 (lanes 6 and 11) or control mouse IgG (lanes 5 and 10). Lane 1 shows a control in the absence of the nuclear extract. White arrowheads indicate the complexes detected (i and ii). A black arrowhead indicates the supershifted complex. The complete sequence of the NFκB probe is shown at the bottom of the figure. The specific nucleotides mutated in the NFκB oligonucleotide are underlined. The gel shown is representative of at least two independent experiments.

were detected in nuclear extracts from both MDCK-Snail and MDCK-CMV cells but the main retarded complex (ii) showed a much lower intensity in MDCK-CMV control cells (Fig. 5). Binding could be competed with an excess of the unlabelled specific NFκB oligonucleotide TGGAATTCCCCA but not with the mutated oligonucleotide TGGAACTCACCA (Fig. 5, lanes 3, 8 and 4, 9, respectively). Inclusion of an anti-p65 antibody supershifted both complexes, indicating that the p65 proteins were involved in binding to the NFκB sequence in these cells (Fig. 5, lanes 6 and 11).

The –389 nt MMP-9 region (shown in construct III, Fig. 2A) contains several potential regulatory elements, including two AP-1 binding sites at nts –505 and –107, shown previously to be important for MMP-9 promoter activity (Fini et al., 1994; Sato and Seiki, 1993). In addition, a further putative AP-1 binding site (GCGGGGTCACT) was found between nts –79 and –69 in the proximal promoter. However, we observed no differences in the binding to AP-1 motifs in nuclear extracts from MDCK-CMV and MDCK-Snail cells in EMSA (data not shown), suggesting that AP-1 elements are not involved in Snail-mediated regulation of MMP-9.

Sp-1 and Ets-1 binding drive transcriptional activity of the minimal MMP-9 promoter

We focused on the activity of the constructs IV-VI to further analyse other Snail-responsive element(s) in the proximal MMP-9 promoter. Both constructs exhibited similar promoter activity that was 2-3 times greater in MDCK-Snail cells or after transient Snail transfection (Fig. 2B and Fig. 3A). However, the shorter construct VI that only contains a putative GATA-1 element and the TATA box was virtually inactive in these cells (Fig. 2B and Fig. 3A). Deletion of the promoter region from nts –97 to –75 was associated with a substantial reduction in the activation of the MMP-9 promoter by Snail. Hence, we wondered whether transcription factor binding sites in this part of the promoter were required for MMP-9 activation by the Snail protein. A computer search of this part of the sequence detected a GC-rich segment with one high-affinity binding site for Sp-1 at nt –81 (GGGCGG; HA), several low affinity Sp1 sites (GGGAGG; LA), and two contiguous putative Ets-1 binding sites (GGAGG) at positions –90 and –85 which overlapped the two low-affinity binding sites for the Sp1 family of transcription factors. We explored the function of the different sites in this region by generating independent mutations in the Ets- and Sp1-consensus sites of the proximal promoter (construct V; Fig. 6A). Mutation of the Ets-1/Sp1 LA sites to GAATGGGAGG (construction $V^{M89/87}$) did not reduce the activity of the promoter but instead increased the activity of the reporter. This result might reflect the functional contribution of both HA Sp-1 and proximal Ets-1 sites that still are present in this construct. In agreement with this proposal, the introduction of mutations on the HA-Sp-1 site between nts –85 and –82 to GGAGGTGATG (construction $V^{M85/82}$) reduced the reporter activity by 85% with respect to the wild-type construct (Fig. 6B). This suggests that both the HA Sp-1 and proximal Ets-1 elements are crucial to sustain the basal activity and the response of MMP-9 promoter to Snail expression.

DNA binding activity in nuclear extracts of MDCK-CMV

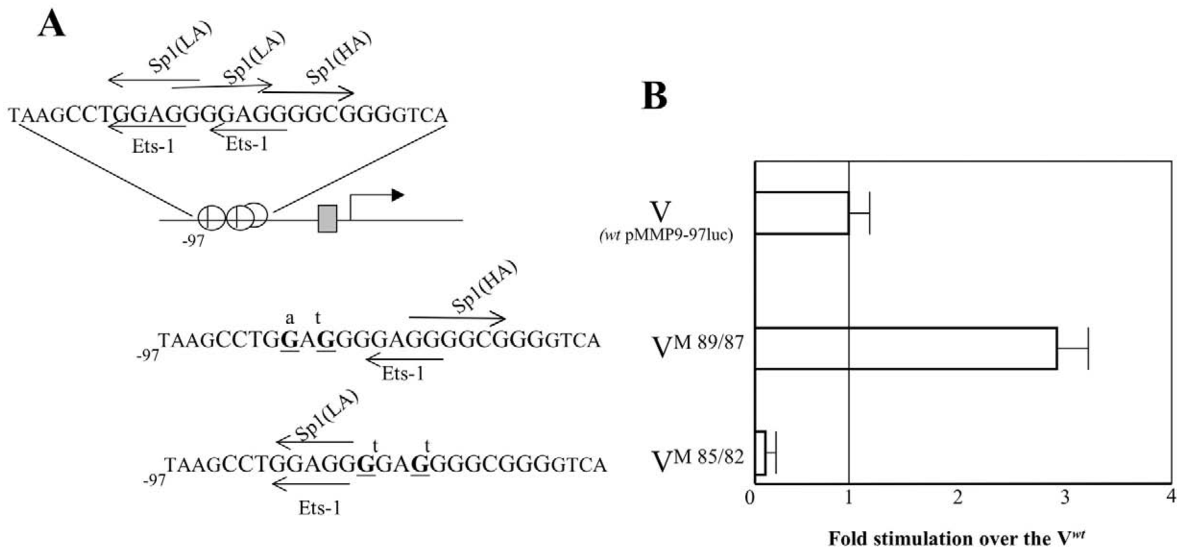


Fig. 6. Ets-1 and Sp-1(HA) elements are responsible for the increase in MMP-9 proximal promoter activity in MDCK-Snail cells. (A) Top, schematic representation of the proximal 5′ region –97 bp from the initiation transcription site, indicating the position of the potential regulatory control elements Ets-1, Sp-1 low affinity (LA) or high affinity (HA) and TATA-like (grey box). Middle and lower sequences: point mutations and putative Ets-1 and Sp-1(LA or HA) binding sites are indicated in each mutant construction. (B) The wild-type pMMP9-97 construct (construct V^{wt}) or mutants (construct $V^{M\ 89/87}$ and $V^{M\ 85/82}$) fused to the *luc* reporter gene, were co-transfected with TK renilla into MDCK-Snail cells. Luciferase activity of each construction was determined and represented as fold stimulation of the activity over the –97 MMP-9 promoter (construct V^{wt}) as the mean±s.d. of three independent experiments.

and MDCK-Snail cells was analysed by EMSA using a labelled probe encompassing nucleotides at positions –85 to –65 from the MMP-9 promoter that includes the HA-Sp-1 and Ets-1 sites. As shown in Fig. 7A, lane 7, a major DNA-protein complex was detected in MDCK-Snail cells in the absence of serum that was absent in MDCK-CMV cells cultured under the same conditions. The specificity of this complex was confirmed by its competition with an excess of the unlabelled wild-type oligonucleotide but not with the mutant (Fig. 7A lanes 8 and 10, respectively). The results presented in Fig. 7B were obtained using a probe encompassing all the Sp-1 and Ets-1 sites (nucleotides at positions –97 to –76) and also show a main retarded complex generated by MDCK-Snail cells compared to MDCK-CMV control cells (Fig. 7B lane 3 and 2 respectively). Indeed, both wild-type probes (encompassing nts –85 to –65 and –97 to –76) from this region of the MMP-9 promoter generated the same complexes (data not shown). Furthermore, to confirm that the indicated complex was attributable to the binding of both the Sp-1 and Ets-1 transcription factors, specific anti-Sp-1 or anti-Ets-1 antibodies were included. Both anti-Ets-1 and anti-Sp1 antibodies lowered the intensity of the complex and the anti-Sp-1 antibody also induced a weak supershifted band visible after long exposure of the gel (data not shown) suggesting that both transcription factors participate in the generation of the specific complex in MDCK-Snail cells.

Oncogenic H-*Ras* (*Ras*V12) synergistically co-operates with Snail in activating MMP-9 transcription

There is strong evidence that the *Ras* oncogene itself is a potent stimulus for MMP-9 transcription and secretion (Bernhard et al., 1995; Gum et al., 1996). Hence, we explored whether activated H-*Ras* collaborates with Snail in inducing MMP-9 promoter activation. MDCK cells were transiently co-transfected with an expression vector encoding an activated H-*Ras* (*Ras*Val12) or a dominant negative version (*Ras*N17). While Snail and *Ras*Val12 alone induced modest promoter activity in construct III (2- and 3.5-fold, respectively), a strong synergistic effect was observed when activated *Ras* and Snail were co-transfected, producing a 20-fold increase in the activity of the MMP-9 promoter (construct III) in MDCK cells (Fig. 8). Furthermore, transient expression of a dominant negative version of *Ras* (*Ras*N17) led to a 60% reduction in Snail-mediated activation of the MMP-9 promoter (Fig. 8A). These results strongly suggest that activated H-*Ras* is required for, and synergistically co-operates with Snail in MMP-9 promoter induction. Furthermore, the proximal AP-1 site at position –107 in construct III might not be the only element implicated in the induction of MMP-9 promoter through H-*Ras*. Deletion of this region (construct V and smaller constructs) did not significantly alter the synergistic co-operation between H-*Ras* and Snail (data not shown), suggesting that the proximal AP-1 motif at nt –79 is also important.

Consistent with the promoter activity, induction of MMP-9 activity in MDCK-Snail cells by activated H-*Ras* (*Ras*Val12) was confirmed as assessed by gelatin zymography analysis (Fig. 8C). In addition, a strong reduction of MMP-9 activity was observed in the presence of a dominant negative version of *Ras* (*Ras*N17).

Multiple signalling pathways regulate Snail-induced MMP-9 secretion

Oncogenic H-*Ras* activates multiple downstream signal transduction pathways, including the Raf/MAPK module and

the PI3K-PKB/Akt pathway implicated in regulating the expression of MMP-9 (Gum et al., 1996). We analysed the *Ras*-activated pathways that might be required for Snail-mediated induction of MMP-9, employing specific low molecular mass inhibitors that selectively block either the MAPK pathway (UO126) or PI3K signalling (wortmannin). The treatment of MDCK-CMV cells with UO126 (10 μM) led to a 30% decrease in the activity of promoter construct III induced by either *Ras*V12 alone, or Snail and *Ras*V12 together (Fig. 8A). In addition, the MAPK inhibitor significantly reduced MMP-9 transcriptional activity (construct III) induced by Snail by 30% (Fig. 9A). Furthermore, treatment with a combination of MAPK and PI3K inhibitors (UO126 and wortmannin) reduced activity by 47% (Fig. 9A).

However, the *jun* amino-terminal kinase (JNK) has also been shown to regulate expression of MMP-9 (Gum et al., 1997). Since JNK, via JNKK, is a downstream effector of the protein kinase MEKK, we asked whether Snail-induced transcriptional activation of MMP-9 was modulated through JNK. The MDCK-CMV cells were exposed to a synthetic inhibitor of JNK signalling (JNK inhibitor II), but this did not reduce the activity of the MMP-9 reporter constructs (Fig. 9A). Neither

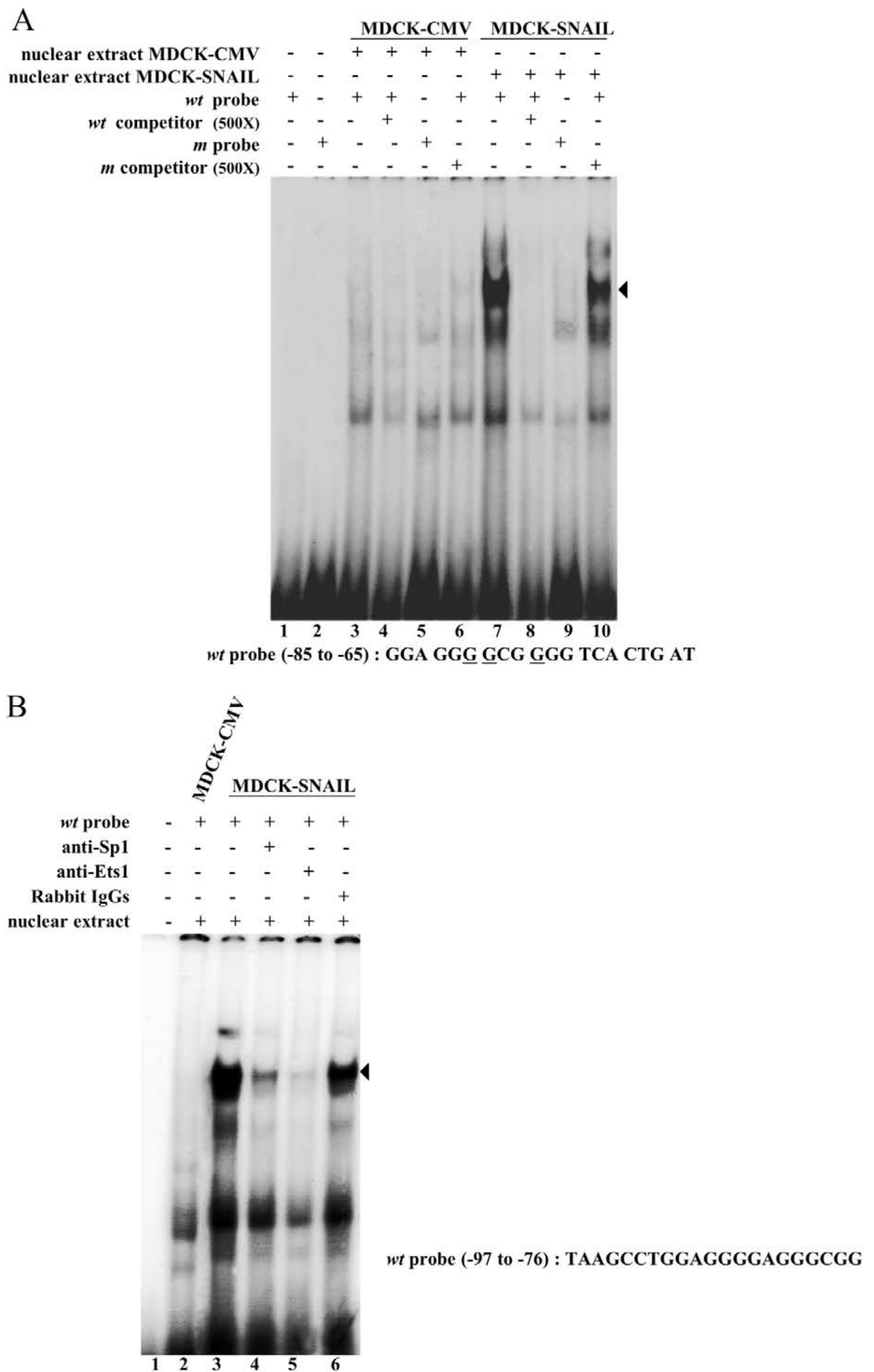


Fig. 7. Nuclear extracts from MDCK-Snail cells contain specific Ets-1 and Sp-1 binding complexes not observed in MDCK-CMV cells. (A) Nuclear extracts from MDCK-CMV and MDCK-Snail cells were analysed by EMSA showing the binding of nuclear proteins to the HA-SP-1 and Ets-1 elements. Nuclear proteins from serum free cultures were incubated with the ^{32}P-labelled wild-type probe encompassing the sequence from nts –85 to –75 of MMP-9 promoter in the absence or presence of 500-fold molar excess of wild-type (lanes 3 and 8) or mutant cold oligonucleotides (lanes 6 and 10). Lanes 1 and 2 show EMSA in which the nuclear extracts were not added. The ^{32}P-labelled mutant probe was used in lanes 5 and 9. Black arrowhead indicates the main complex detected. The complete sequence of the wild-type probe is shown at the bottom of the figure in which the mutated nucleotides are underlined. The gel shown is representative of at least two independent experiments. (B) Nuclear extracts of MDCK-CMV (lane 2) and MDCK-Snail cells (lanes 3-6) were analysed in band-shift assays. The ^{32}P-labelled wild-type (wt) oligonucleotide encompassing the sequence from nts –97 to –75 of the MMP-9 promoter is shown at the bottom. Nuclear extracts were incubated with either an anti-Sp-1 (lane 4) or anti-Ets-1 (lane 5) antibodies, or control rabbit IgG (lane 6). A black arrowhead indicates the main complex detected, the intensity of which is lowered by both antibodies. The gel shown is representative of four independent experiments.

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did an inhibitor of the p38 pathway, SB 203580, modify Snail-induced activity of the MMP-9 promoter (Fig. 9A).

Increased levels of active ERKs (phospho-p42 and phospho-p44) and active Akt in MDCK-Snail cells were confirmed by western blots (Fig. 9B,C). In addition, an antibody that recognised both the phosphorylated and unphosphorylated forms of Sp-1, detected increased levels of both forms of Sp-1 protein in Snail-expressing cells but not in MDCK-CMV (Fig. 9D). Phosphorylation of Sp-1 by ERK provokes an increase in the transcriptional activity of Sp1-dependent genes (Black et al., 2001; Reisinger et al., 2003). To evaluate whether phosphorylation of Sp-1 through Erk may influence the DNA-binding activity of this transcription factor to the proximal MMP-9 promoter, we performed EMSA experiments with nuclear extracts of MDCK-Snail cells expressing a dominant negative mutant of MEK (MEK-DN) with the –97 to –76 nt probe. As shown in Fig. 9E, transfection of this MEK mutant provoked a clear reduction in the intensity of complexes. As expected, anti-Erk1/2 antibodies were able to detect a decrease in the phosphorylated forms of Erk after the expression of MEK-DN (Fig. 9E, lower panels). These results clearly demonstrate that phosphorylated Sp-1 is recruited to the MMP-9 promoter following activation of the Erk1/Erk2 pathway.

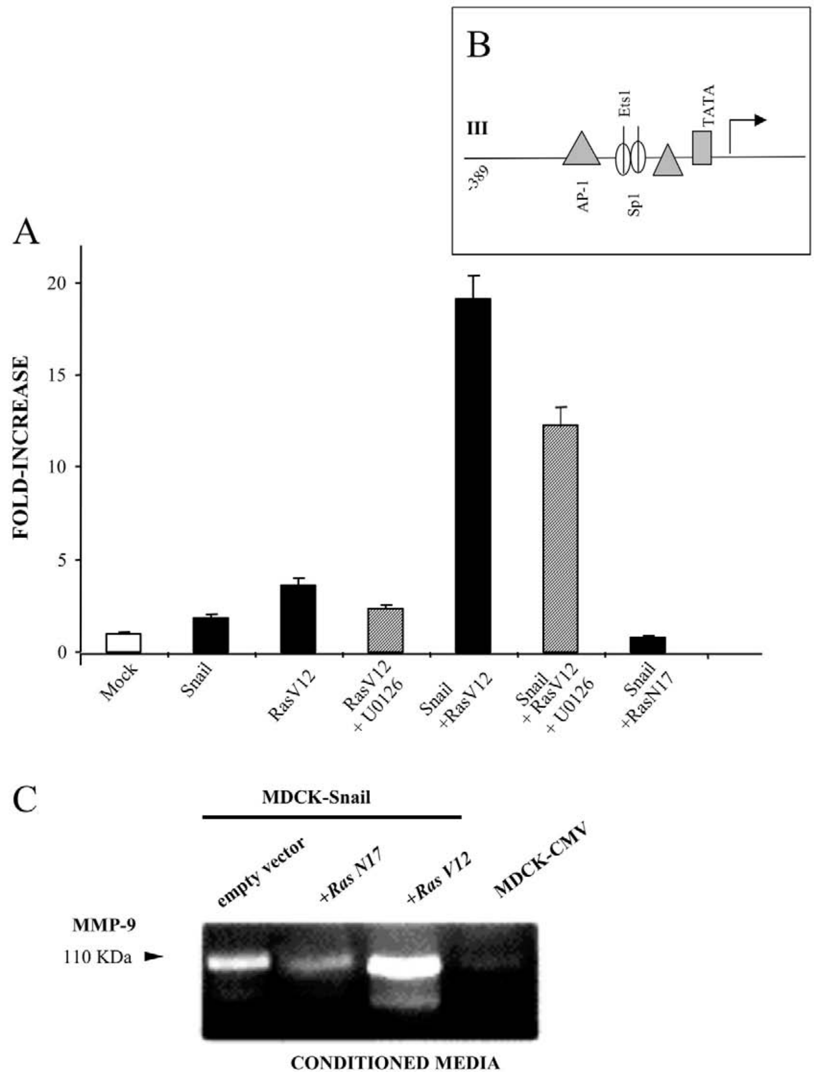

Fig. 8. Induction of MMP-9 promoter activity and gelatinolytic expression by synergistic co-operation of oncogenic *Ras*Val12 and Snail. (A) MDCK-CMV cells were transiently co-transfected with the pMMP9-392luc reporter construct and the indicated combinations of oncogenic *Ras* (*Ras*Val12), Snail, a dominant negative *Ras* (*Ras*N17) or the pCDNA3 expression vector (Mock), as well as TK renilla. After transfection, the cells were treated for an additional 5 hours with UO126 inhibitor (10 μM) or DMSO, prior to determining luciferase activity. The data are expressed as the mean±s.d. of the relative normalised luciferase values from three independent experiments. (B) Schematic representation of the proximal 5′ region examined in these experiments. The pMMP9-389luc (construct III) contains 389 bp from the initiation transcription site and the positions of potential regulatory control elements are indicated. (C) The effects of Snail and *Ras* oncogene were analysed in the conditioned media by zymography in gelatin-embedded SDS polyacrylamide gels. MDCK-Snail cells were transiently transfected with oncogenic *Ras* (*Ras*V12), a dominant negative *Ras* (*Ras*N17) or the empty plasmid and media were collected after 24 hours. Conditioned medium from MDCK-CMV cells was also included as control.

Discussion

The recent identification of Snail as a strong repressor of E-cadherin and a potent mediator of EMT (Cano et al., 2000), suggests that Snail may act on other target genes that contribute to the phenotypic changes associated with this process (Guaita et al., 2002; Vega et al., 2004). One of the hallmarks of the EMT, both in embryogenesis and in pathological situations such as tumour invasion, is the enhanced capacity to invade and migrate through the basal membranes. As such, a functional link between E-cadherin and the MMPs has been established (Llorens et al., 1998; Nawrocki-Raby et al., 2003). Indeed, Snail has recently been shown to induce MMP-1, MMP-2, MMP-7 and MT1-MMP in HepG2 and Huh-7 hepatocellular cell lines and squamous cell carcinomas (Miyoshi et al., 2004; Yokoyama et al., 2003). We show here that Snail expression induces an increase in the gelatinolytic activity of MMP-9, accompanied by a significant increase in MMP-9 mRNA levels. Secondly, we show that the induction of MMP-9 promoter activity by Snail is partly mediated through multiple elements located in the region upstream from the transcription start site.

When human and mouse MMP-9 gene sequences are compared the major binding sites for AP-1, Sp-1 and NFκB are conserved in both genomes, as is an E-box element located at nt –648. Although this element seems to be involved in Snail-mediated induction of MMP-9 expression through the activation of the large –1.17 nt MMP-9 promoter construct, we have not observed specific binding to this element in vitro in MDCK-Snail cells, probably because it differs from the consensus Snail binding element. Nevertheless, the specific

binding of factors to elements such as NFκB, Sp-1 and Ets-1 is augmented in Snail-expressing cells.

The NFκB site is indispensable for TGF-β-mediated transcriptional downregulation of MMP-9 in monocytes/macrophages (Ogawa et al., 2004) as well as for both basal and PMA-induced transcription, acting together with AP-1 (Sato and Seiki, 1993). The mechanism leading to the activation of NFκB is not well understood in our system, although it might be mediated, at least in part, by PKB/AKT signalling (Ozes et al., 1999; Romashkova and Makarov, 1999). Nevertheless, it is worth mentioning that the amount of p65 protein was not altered by Snail expression in MDCK cells (data not shown), suggesting that differential binding to NFκB sites probably results from the activation of NFκB-p65 transcription factors. *Trans*-acting activity of the MMP-9 promoter-bound transcription factors, appears to be crucial but not exclusive for MMP-9 expression. Indeed, the enforced expression of the metastases-associated gene *MTA1* has been shown to reduce basal and PMA-induced MMP-9 protein and mRNA levels without altering the occupancy of these sites through a mechanism that is both dependent on and independent of histone deacetylation (Yan et al., 2003). Irrespective of the specific mechanisms, our results are consistent with previous reports showing that NFκB expression is associated with more malignant phenotypes in squamous cell skin carcinomas (Dong et al., 1999) and breast carcinoma (Sovak et al., 1999). Moreover, increased NFκB activity and signalling regulates MMP-9 expression (Farina et al., 1999; Kondraganti et al., 2000), and has a dramatic impact on metastatic potential (Andela et al., 2000) and invasion through Matrigel (Huang et al., 2001).

In addition to the aforementioned elements, a region lying upstream of the AP-1/TRE, the GGGGAGGGG motif, and related sequences are also conserved in the mouse genome (from nts –87 to –79). However, an important difference in the region located between the proximal AP-1 site (at nt –107) and

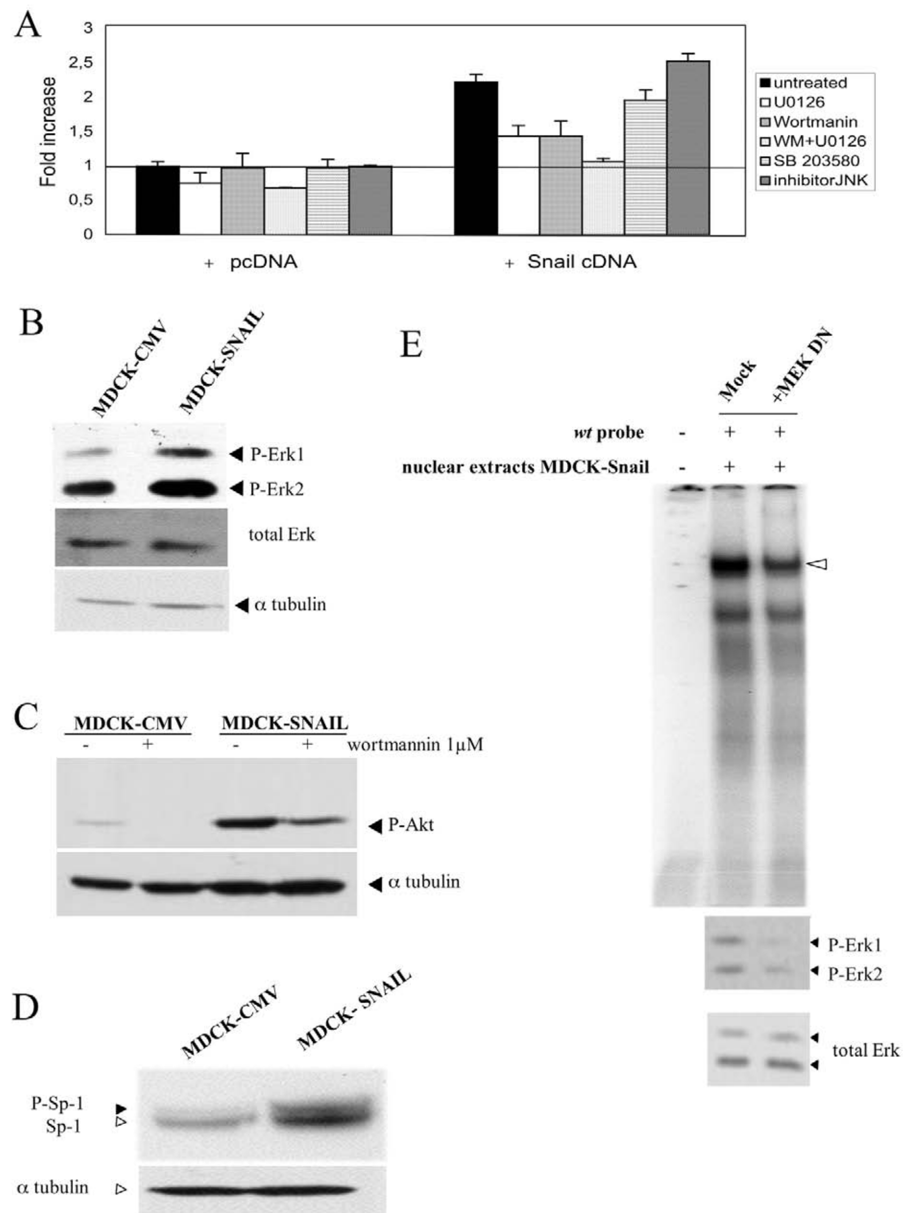

Fig. 9. MAPK and PI3K signalling pathways are involved in the induction of MMP-9 proximal promoter by Snail. (A) MMP-9 promoter activity was measured in MDCK-CMV cells by using the reporter construct pMMP9-389luc in the absence or presence of the Snail expression vector, and in the presence or absence of the following inhibitors: UO126 (10 μM); wortmannin (40 nM); SB 203580 (10 μM) or JNK inhibitor II (100 nM). Control cells (untreated) were unstimulated and treated with DMSO alone. Promoter activity is expressed relative to the value obtained in MDCK-CMV untreated cells and results are expressed as the mean±s.d. of three independent experiments. (B,C) Effect of Snail on Erk (B) and Akt (C) phosphorylation. Total cellular protein was extracted from MDCK-CMV and MDCK-Snail untreated cells and cells treated with wortmannin (C). 150 μg were analysed by western blotting using specific antibodies for the phosphorylated forms of these protein kinases. α-tubulin was used as a loading control. (D) Cell extracts (30 μg) from MDCK-CMV and MDCK-Snail cells were used in western blots to determine Sp-1 phosphorylation status using a specific antibody that recognizes the phosphorylated and unphosphorylated isoforms. α-tubulin was used as a loading control. One representative experiment out of five is shown. (E) Nuclear extracts of MDCK-Snail cells cultured for 24 hours in serum-free medium were analysed by EMSA after transient transfection with MEK-DN expression vector or empty plasmid. The ^{32}P-labelled wild-type (wt) oligonucleotide encompassing the sequence from nts –97 to –76 is the same as that in Fig. 7B. The same nuclear extracts were analysed by western blotting using specific antibodies for the phosphorylated forms of Erk protein kinases. Anti-total Erk antiserum was used in the same membrane as a loading control.

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the TATA box, was that this GC-rich motif (GGGGAGGGGCGGGG) in the mouse gene corresponds to a GT-box (GGGGTGGGG) in the human gene. Substitution of a GT box for a GA box, has been reported to have dramatic effects on the response of the MMP-9 promoter to *Src* (Sato et al., 1993). Moreover, GT-box sequences have been mapped as RCEs (retinoblastoma control elements) and they are present in the promoter regions of *Myc*, *Fos*, transforming growth factor β1 (*Tgfb1*), insulin-like growth factor 2 (*Igf2*) and many other genes (Kim et al., 1991). Similarly, the GC-rich sequences in the *Jun*B and *EGR-1* gene promoters have also been identified as RCEs. While it has not yet been demonstrated that the RCE stimulates the MMP-9 promoter, Sp-1 might bind to the RCE and activate transcription of human MMP-9 (Kim et al., 1992). Indeed, Rb positively regulates RCE-mediated transcriptional activity by Sp-1. Similarly, the proximal region between –97 bp and the TATA box sustained Snail-induced stimulation of MMP-9 promoter activity and we explored the contribution of several putative elements within this region.

We found evidence that the elements located within the –97 to –75 nt region are sufficient for minimal activation of the MMP-9 promoter by Snail. However, while the AP-1 motif GCGGGGTCACT (from nts –79 to –69) overlapping the GC box might contribute to the MMP-9 response, the presence of Snail did not alter binding to this element in vitro (data not shown). In contrast, Snail expression increased the binding to the Ets-1 and Sp-1 elements in vitro, accounting for the increased activity of the MMP-9 proximal promoter. We suggest that binding of both factors exerts a co-operative effect on promoter activation through the MAPK- and PI3K-dependent signalling pathways. Indeed, disruption of ERK and PI3K-dependent signalling inhibits the Snail-mediated increase in MMP-9 promoter activity in MDCK cells. Activated ERK kinases may phosphorylate either Ets-1or Sp-1 proteins enhancing transcription of the genes responsive to these elements (Black et al., 2001; Milanini-Mongiat et al., 2002; O'Neill et al., 1994). Indeed, increased phosphorylation of Sp-1 was detected in MDCK-Snail cells.

The constitutive activation of ERK 2 kinase in MDCK-Snail cells may activate the MMP-9 proximal promoter through different effectors. These results are consistent with reports showing that ERK 1/2 increases the activation and activity of the MMP-9 promoter via MEK1 (Gum et al., 1996; Gum et al., 1997). However, since specific inhibitors only reduced activity by 30%, it seems likely that other signal pathways also modulate the effects of Snail on MMP-9 promoter activity. Indeed, the PI3K pathway co-operates with ERK 2 in activating the MMP-9 promoter in MDCK cells in the presence of Snail. Taken together, our results support a role for the MAPK and PI3K pathways in Snail-mediated MMP-9 induction. Both pathways participate in EMT and an invasive MDCK cell phenotype has been reported in cells with activated MEK1 following TGF-β treatment (Janda et al., 2002; Peinado et al., 2003), as well as with an inducible form of Raf (Raf-ER) which leads to the autocrine production of TGF-β (Lehmann et al., 2000). Furthermore, the MAPK and PI3K signalling pathways are involved in the activation of Snail transcription (Barbera et al., 2004; Peinado et al., 2003) suggesting that it might contribute to sustain the activation of the MMP-9 gene in MDCK-Snail cells. Taken together, Snail expression confers a double selective advantage to cells, making them resistant to cell death (Vega et al., 2004) and enabling them to degrade the major components of basement membranes, which are requisites for malignant cells to disseminate and form metastasis.

Significantly, we also observed a synergism between *Ras* and Snail in the induction of MMP-9 promoter activity. This may also be the result of augmenting the activity of the MAPK and PI3K pathways, since *Ras* can stimulate both signalling proteins (Karin, 1995; Webb et al., 1998). Importantly, ERKs also increase the synthesis and/or activity of several transcription factors, including members of the AP-1 families and *Fos* (Gille et al., 1992), as well as Ets and Sp-1 (Bouwman and Philipsen, 2002; O'Neill et al., 1994). Participation of the AP-1 factors seems only to be required for *Ras*-mediated induction of the MMP-9 promoter, suggesting that it contributes to the synergistic activation of the promoter by *Ras* and Snail.

Taken together, our results confirm previous reports of elements that control MMP-9 expression, including the AP-1, NFκB, Sp-1 and Ets-1 sites within the –670 bp region upstream to the transcription start site (Farina et al., 1999; Sato et al., 1993; Takahra et al., 2004; Troussard et al., 2000; Yan et al., 2004). Moreover, they support an important role for activated MAPK on the activation of the proximal MMP-9 promoter, mainly within the –97 bp region upstream of the transcription start site. While the involvement of MAPK and PI3K in EMT is well documented (Janda et al., 2002; Montesano et al., 1999; Schramek et al., 1997), the nature of the transduction pathways used by Snail and that lead to the activation of MAPK remain unknown. These pathways appear to be particularly significant and might enable Snail to co-ordinate the events that lead to the expression of genes required for the EMT, and that facilitate tumour progression.

In conclusion, MMP-9 transcription is activated in response to Snail expression concomitantly with the activation of the MAPK and PI3K signalling pathways. The increased expression of this matrix metalloproteinase may explain, at least in part, the invasive properties acquired by Snail-expressing cells in the context of the EMT as well as in tumour progression.

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