

p38 maintains E-cadherin expression by modulating TAK1–NF- κ B during epithelial-to-mesenchymal transition

Raffaele Strippoli^{1,2}, Ignacio Benedicto^{3,4}, Miguel Foronda¹, Maria Luisa Perez-Lozano³, Sara Sánchez-Perales¹, Manuel López-Cabrera^{3,4,5} and Miguel Ángel del Pozo^{1,*}

¹Integrin Signaling Laboratory, Department of Vascular Biology and Inflammation, Centro Nacional de Investigaciones Cardiovasculares (CNIC), Melchor Fernández Almagro 3, 28029 Madrid, Spain

²Bambino Gesù Children Hospital, Piazza Sant'Onofrio 4, 00165 Rome, Italy

³Unidad de Biología Molecular, Hospital Universitario de la Princesa, Instituto de Investigación Sanitaria Princesa (IP), 28006 Madrid, Spain

⁴CIBER-ehd, Instituto de Salud Carlos III, 28029, Madrid, Spain

⁵Centro de Biología Molecular Severo Ochoa, CSIC-UAM, Cantoblanco, 28049, Madrid, Spain

*Author for correspondence (madelpozo@cnic.es)

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Summary

Epithelial-to-mesenchymal transition (EMT) of peritoneal mesothelial cells is a pathological process that occurs during peritoneal dialysis. EMT leads to peritoneal fibrosis, ultrafiltration failure and eventually to the discontinuation of therapy. Signaling pathways involved in mesothelial EMT are thus of great interest, but are mostly unknown. We used primary mesothelial cells from human omentum to analyze the role of the p38 MAPK signaling pathway in the induction of EMT. The use of specific inhibitors, a dominant-negative p38 mutant and lentiviral silencing of p38 α demonstrated that p38 promotes E-cadherin expression both in untreated cells and in cells co-stimulated with the EMT-inducing stimuli transforming growth factor (TGF)- β 1 and interleukin (IL)-1 β . p38 inhibition also led to disorganization and downregulation of cytokeratin filaments and zonula occludens (ZO)-1, whereas expression of vimentin was increased. Analysis of transcription factors that repress E-cadherin expression showed that p38 blockade inhibited expression of Snail1 while increasing expression of Twist. Nuclear translocation and transcriptional activity of p65 NF- κ B, an important inducer of EMT, was increased by p38 inhibition. Moreover, p38 inhibition increased the phosphorylation of TGF- β -activated kinase 1 (TAK1), NF- κ B and I κ B α . The effect of p38 inhibition on E-cadherin expression was rescued by modulating the TAK1–NF- κ B pathway. Our results demonstrate that p38 maintains E-cadherin expression by suppressing TAK1–NF- κ B signaling, thus impeding the induction of EMT in human primary mesothelial cells. This represents a novel role of p38 as a brake or ‘gatekeeper’ of EMT induction by maintaining E-cadherin levels.

Key words: E-cadherin, EMT, NF- κ B, p38, Snail, TAK1, TGF- β 1, Twist

Introduction

EMT is a complex, stepwise phenomenon that occurs during embryonic development and tumor progression (Thiery et al., 2009), and is also associated with chronic inflammatory and fibrogenic diseases affecting lung, liver and the peritoneum of patients undergoing peritoneal dialysis (Kalluri and Weinberg, 2009; Aroeira et al., 2007). During dialysis, the peritoneum is exposed to continuous inflammatory stimuli such as hyperosmotic, hyperglycemic and acidic dialysis solutions, as well as episodes of peritonitis and hemoperitoneum, which might cause acute and chronic inflammation and progressively lead to fibrosis, angiogenesis and hyalinizing vasculopathy. Our previous work demonstrated that effluent-derived mesothelial cells (MCs) from peritoneal dialysis patients show phenotypic changes, reminiscent of EMT, which are associated with the time of peritoneal dialysis treatment and with episodes of peritonitis or hemoperitoneum (Yanez-Mo et al., 2003). Moreover, the appearance of EMT signs correlates with structural and functional deterioration of the peritoneal membrane (Aroeira et al., 2007).

EMT is characterized by the disruption of intercellular junctions, replacement of apical-basolateral polarity with front-to-back polarity, and acquisition of migratory and invasive phenotypes.

Cells that have undergone EMT also acquire the capacity to produce extracellular matrix (ECM) components and a wide spectrum of inflammatory, fibrogenic and angiogenic factors. EMT is triggered by an interplay of extracellular signals, including components of the ECM, as well as soluble growth factors and cytokines, including members of the transforming growth factor (TGF)- β and fibroblast growth factor families, epidermal growth factor and hepatocyte growth factor (Thiery et al., 2009).

The molecular mechanisms controlling the establishment and progression of EMT appear to be multifold and cell-type specific. One of the key events in EMT is the disruption of cadherin junctions between epithelial cells, and a pivotal role is played by families of transcription factors that repress E-cadherin expression, such as Snail, ZEB and basic helix-loop-helix (bHLH) families (Peinado et al., 2007). The expression of these transcription factors is controlled by a complex network of signaling molecules, including SMADs, integrin-linked kinase, phosphatidylinositol 3-kinase, mitogen-activated protein kinases (MAPKs), glycogen synthase kinase 3 β , and nuclear factor κ B (NF- κ B) (Thiery et al., 2009; Zavadil and Bottinger, 2005).

MAPKs are serine/threonine kinases that play important roles in a vast array of pathophysiological processes. The family is divided

into three main subfamilies: extracellular regulated kinase (ERK), Jun N-terminal kinase (JNK) and p38. All are characterized by the presence of a typical activation module and a conserved activation domain (Chang and Karin, 2001). ERK1 and ERK2 are activated by mitogenic stimuli, whereas JNK and p38, also called stress-activated protein kinases (SAPKs), are activated by environmental and genotoxic stresses (Wagner and Nebreda, 2009). Besides being a central mediator of the inflammatory and stress response, p38 plays an important role in non-inflammatory processes such as cell-cycle regulation and cell differentiation (Cuenda and Rousseau, 2007). Once activated, p38 phosphorylates a wide array of substrates in the cytoplasm and in the nucleus, thus regulating gene expression, cell cycle and cellular polarization. The induction or activation of phosphatases is an important mechanism of crosstalk between MAPKs, and allows p38 to regulate the activity of ERK and JNK (Junttila et al., 2008; Perdiguer et al., 2007). Studies into the roles of MAPK families in the genesis of EMT have produced conflicting results, due to the heterogeneity of the cellular models and the different experimental approaches used. There is compelling evidence that ERKs drive EMT in many experimental systems (Zavadil and Bottinger, 2005), and our previous work has demonstrated the role of ERKs in E-cadherin downregulation and EMT induction (Strippoli et al., 2008). However, the role of SAPKs is less studied, although some reports indicate that JNK is an EMT inducer, also in MCs (Alcorn et al., 2008; Liu, Q. et al., 2008). p38 appears to promote EMT during development and in tumors (Zavadil and Bottinger, 2005; Zohn et al., 2006; Liu, Y. et al., 2008); however, the role of p38 in EMT during chronic inflammatory disease has not been analyzed. Here, we demonstrate that p38 promotes E-cadherin expression by suppressing TGF- β -activated kinase 1 (TAK1)–NF- κ B signaling, thus impeding the induction of EMT in human primary mesothelial cells.

Results

Inhibition of p38 MAPK represses E-cadherin and cytokeratin expression in primary MCs

The main inducers of EMT in MCs *in vivo* are thought to be combinations of inflammatory and profibrotic cytokines, such as TGF- β 1 and IL-1 β . We have shown that this combination can induce a genuine EMT in primary omentum-derived MCs (Yañez Mo et al., 2003) and described the role of ERK MAPK in EMT induction in MCs (Strippoli et al., 2008). To analyze the role of SAPKs in E-cadherin expression, we pretreated MCs with specific inhibitors of p38 and JNK before stimulation with a combination of TGF- β 1 (0.5 ng/ml) and IL-1 β (2 ng/ml) for 24 hours (T/I stimulation). These stimuli are able to induce a sustained activation of ERK (Strippoli et al., 2008) and p38 (supplementary material Fig. S1A). Moreover, p38 is stably activated in quiescent MCs and, differently from ERK, its activation levels are increased upon cellular confluency in MCs and in other experimental systems (supplementary material Fig. S1B) (Faust et al., 2005). As previously described, inhibition of ERK signaling with U0126 increased basal E-cadherin expression and limited its downregulation upon cytokine treatment (Fig. 1A) (Strippoli et al., 2008). On the other hand, treatment with p38 inhibitor SB203580 markedly reduced E-cadherin expression in both untreated and cytokine-treated cells (Fig. 1A), whereas inhibition of JNK with SP600125 had some effect in limiting E-cadherin downregulation (Fig. 1A). The effects of p38 inhibition were confirmed by using BIRB 796 (Pargellis et al., 2002), a more specific p38 inhibitor (Fig. 1A). Similar results were obtained in MCs infected with a

retroviral vector encoding p38AGF, a non-phosphorylatable and inactive mutant form of p38 (Fig. 1B). Specific blockade of p38 activity with the p38AGF construct was confirmed in cells treated with sodium arsenite, a p38 activator (Westermarck et al., 2001) (supplementary material Fig. S2). The reduction in E-cadherin expression upon p38 inhibition was also demonstrated by infecting primary omentum-derived MCs with two specific lentiviral vectors encoding small hairpin RNA (shRNA) specific for p38 α (Fig. 1C), and lentiviral silencing of p38 expression led to the disappearance of E-cadherin from cell membrane junctions (Fig. 1D; supplementary material Fig. S3A). Inhibition of p38 with pharmacologic inhibitors (Fig. 1E) or by lentiviral-mediated shRNA silencing (Fig. 1F) also reduced the expression of E-cadherin-encoding mRNA. On the other hand, treatment of MCs with sodium arsenite led to blockade of T/I-induced E-cadherin downregulation (Fig. 2A). Confocal microscopy analysis showed increased membrane localization of E-cadherin upon treatment with sodium arsenite in cells stimulated with T/I (Fig. 2B; supplementary material Fig. S3B). These results strongly suggest that p38 promotes E-cadherin expression and plasma membrane localization in human primary MCs.

Pharmacological inhibition of p38 also reduced the expression of cytokeratin, another epithelial marker whose downregulation is often associated with EMT, and resulted in a disorganized cytokeratin skeleton (Fig. 3A,B). The expression of ZO-1, a component of tight junctions, was also reduced upon p38 inhibition (Fig. 3B). Confocal analysis showed an altered plasma membrane–cytosolic distribution of ZO-1 in cells treated with p38 inhibitors (Fig. 3C; supplementary material Fig. S3C). Treatment with p38 inhibitors in combination with T/I stimulation led to the appearance of cells with a fibroblastoid shape, and which no longer expressed ZO-1 at the plasma membrane cell edges (Fig. 3C, arrows). On the other hand, the expression of vimentin, a mesenchymal marker, increased upon p38 inhibition (Fig. 3B; supplementary material Fig. S4). These results suggest that p38 inhibition might oppose the expression of different epithelial proteins during EMT induction, while increasing levels of mesenchymal markers.

Blockade of p38 decreases expression of Snail1 and induces expression of Twist

We next analyzed the role of p38 in the expression of transcription factors known to directly regulate E-cadherin expression in our experimental conditions. We focused first on Snail1, which is a main regulator of E-cadherin expression and is upregulated upon stimulation with TGF- β 1 plus IL-1 β in our experimental system (Yañez Mo et al., 2003). Stimulation of primary MCs with T/I induced a rapid increase in Snail1-encoding mRNA expression that was inhibited by pretreatment with SB203580 (Fig. 4A), and also by pretreatment with the ERK pathway inhibitor U0126, as previously reported (Strippoli et al., 2008). This effect was also reflected in reduced Snail1 protein expression detected by western blot (Fig. 4B) and by immunofluorescence (Fig. 4C). Interestingly, simultaneous pretreatment with SB203580 and U0126 caused a stronger inhibition of the induction of mRNA encoding Snail1. We analyzed whether this effect was due to an involvement of mitogen- and stress-activated kinases 1 and 2 (MSK1/2), which are activated by both ERK and p38 (Vermeulen et al., 2009). However, pretreatment with H89, an inhibitor of MSK1/2 and protein kinase A, ruled out this hypothesis (Fig. 4D). Because increased expression of Snail1 cannot account for decreased E-cadherin upon p38 inhibition, we analyzed the effect of p38 inhibitors on the expression

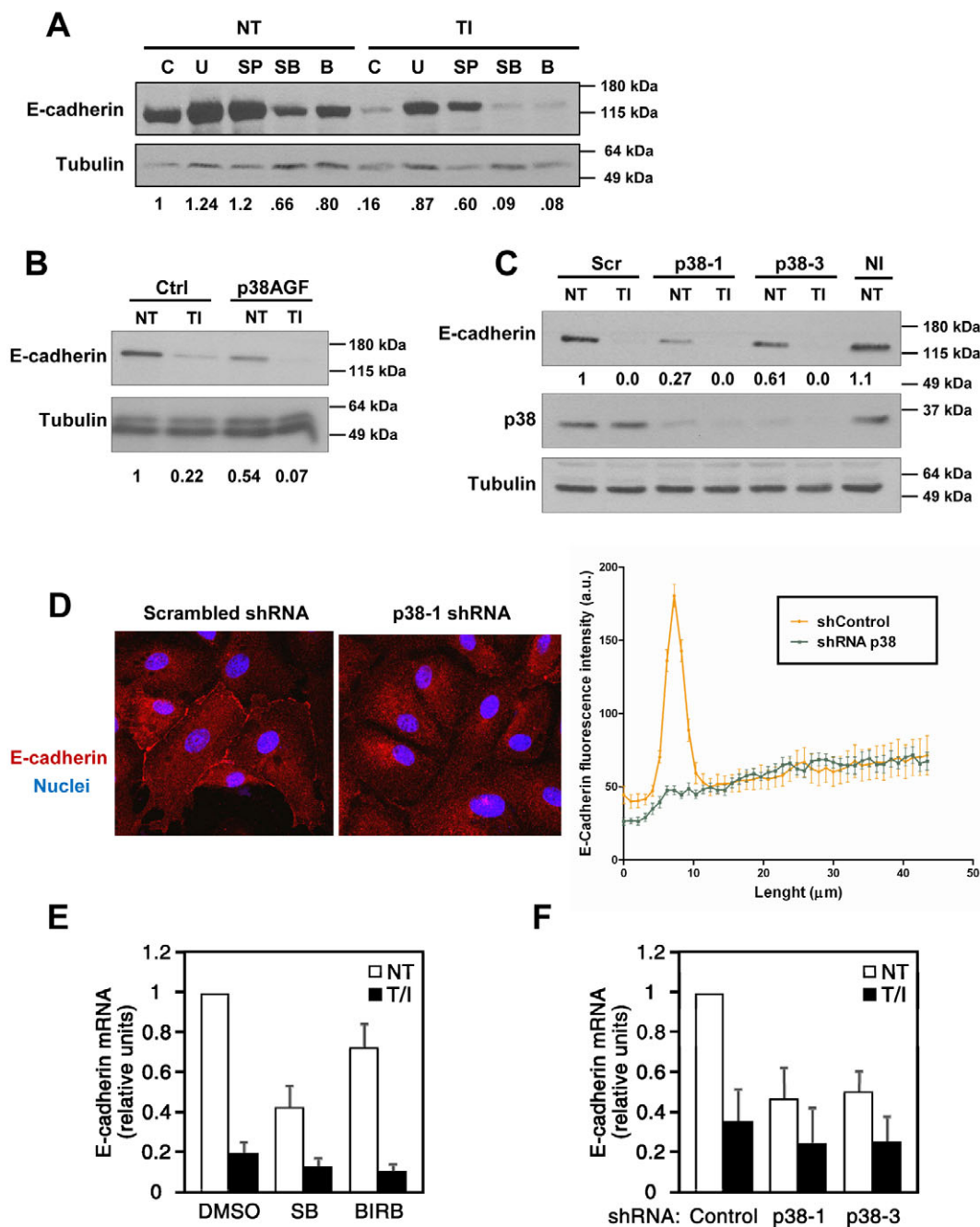


Fig. 1. p38 MAPK controls E-cadherin levels in omentum-derived MCs in resting conditions or upon TGF- β 1 and IL-1 β co-stimulation. (A) Western blots showing the expression of E-cadherin in total lysates of MCs pretreated with DMSO (C), 20 μ M U0126 (U), 10 μ M SP600125 (SP), 10 μ M SB203580 (SB) or 250 nM BIRB 796 (B), and then either not treated (NT) or treated with 0.5 ng/ml TGF- β 1 in combination with 2 ng/ml IL-1 β for 24 hours (TI). Expression of γ -tubulin was detected as a loading control. Data are representative of five independent experiments. (B) Western blots showing the expression of E-cadherin in total cell lysates of MCs infected with a Cop Green-tagged retrovirus encoding p38AGF, or with empty virus (Ctrl). Cells were treated with T/I for 48 hours. Data are representative of three independent experiments. (C) Western blots showing the expression of E-cadherin in total cell lysates of MCs infected with lentiviral vectors encoding specific p38-targeting shRNAs (clones p38-1 and p38-3). Scr, cells infected with control shRNA virus; NI, non-infected cells. p38 expression was detected to verify the silencing, and γ -tubulin was detected as a loading control. Data are representative of three independent experiments. (D) Left: confocal immunofluorescence of E-cadherin expression and localization in control (scrambled) and p38-silenced methanol-fixed MCs. Cell nuclei are shown in blue (Hoechst 33342) and E-cadherin immunofluorescence is shown in red. Representative images are shown of three independent experiments. Right: quantification of the experiment described above (see supplementary material Fig. S3A). (E) Effect of pharmacologic inhibition of p38 on E-cadherin-encoding mRNA expression in MCs. Cells were pretreated with 10 μ M SB203580, 250 nM BIRB 796 or vehicle (DMSO) and stimulated with T/I. mRNA was amplified from total RNA by quantitative RT-PCR; histone H3-encoding mRNA expression was used for normalization. Bars represent means \pm s.e.m. of duplicate determinations in three independent experiments. (F) Effect of p38 shRNA silencing on E-cadherin-encoding mRNA expression in MCs. Cells were infected with lentiviral vectors as in C and stimulated as indicated. Quantitative RT-PCR was performed as in E; bars represent means \pm s.e.m. of duplicate determinations in three independent experiments.

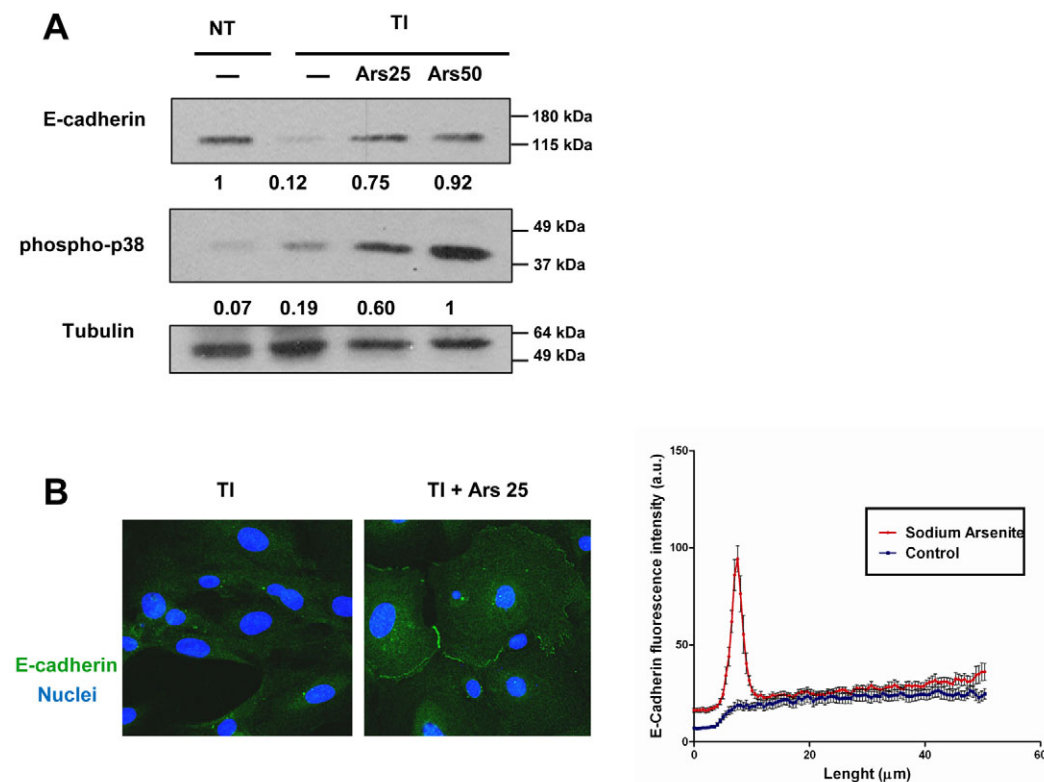


Fig. 2. Effect of sodium arsenite on E-cadherin expression in MCs.

(A) Western blot showing expression of E-cadherin in non-stimulated (NT) and stimulated (TI) MCs. Cells were treated with 0, 25 or 50 μM sodium arsenite (–, Ars25 and Ars50, respectively) for 1 hour prior to stimulation with T/I for 24 hours. Phospho-p38 is shown in the middle panel, and expression of γ-tubulin was detected as a loading control. Representative experiment shown is one of three performed. (B) Left: confocal immunofluorescence of E-cadherin expression and localization in MCs treated with sodium arsenite. Cells were treated as in A. Only cells stimulated with T/I are shown. Cell nuclei are shown in blue (Hoechst 33342) and E-cadherin immunofluorescence is shown in green. Representative experiment shown is one of two performed. Right: quantification of the results (see supplementary material Fig. S3B).

of the zinc finger transcription factors Twist, ZEB1, ZEB2, E47 and Slug. Pretreatment with SB203580 or BIRB 796 had no significant effect on ZEB1 and ZEB2, E47 and Slug, but increased the expression of the bHLH protein Twist (Fig. 4E). The role of p38 on Twist expression was also confirmed by p38 silencing (Fig. 4F). p38 thus has opposing actions on the expression of Snail1 and Twist in MCs.

p38 inhibition increases NF-κB nuclear translocation, DNA binding and transcriptional activity in MCs

NF-κB plays a major role in EMT induction in many experimental settings, including MCs (Huber et al., 2004; Strippoli et al., 2008; Solanas et al., 2008). Moreover, Dorsal, a *Drosophila* homolog of p65 NF-κB, has been shown to control the transcription of Snail and Twist during development and innate immunity in *Drosophila* (Furlong et al., 2001), indicating a role in the expression of Snail and bHLH factors; and p38 activity can, depending on the experimental conditions, enhance or limit NF-κB function (Orr et al., 2005; Gazel et al., 2008). Pretreatment of MCs with SB203580 or BIRB 796 increased the intensity of NF-κB nuclear staining induced by cytokine treatment (Fig. 5A). To analyze NF-κB interaction with specific DNA sequences upon p38 inhibition, we performed a p65 NF-κB binding assay (supplementary material Fig. S5) using MeT5A cells, an untransformed MC line widely used in peritoneal MC research (Rampino et al., 2001). BIRB 796 pretreatment moderately increased NF-κB binding to a specific probe both in unstimulated cells and upon T/I stimulation (Fig. 5B). To test the effect of p38 on NF-κB transcriptional activity, we transfected MeT5A cells with a luciferase reporter construct containing multiple NF-κB binding sites (KBF-luc). Pretreatment of these cells with BIRB 796 increased basal and cytokine-induced luciferase activity (Fig. 5C). This experiment was reproduced in MeT5A cells infected with the p38AGF dominant-negative

construct (Fig. 5D). These results strongly support a role for p38 in limiting NF-κB activity in MCs during EMT.

p38 inhibition enhances phosphorylation of TAK1 on Thr187 and of p65 NF-κB on Ser536, and reduces PP2A phosphatase activity in cytokine-stimulated MCs

We next examined the possible role of TAK1 in p38-regulated NF-κB activation. TAK1 plays a major role in the activation of NF-κB and MAPK pathways in response to TGF-β1 and IL-1β (Shim et al., 2005). Pharmacologic inhibition of p38 before T/I stimulation increased the phosphorylation of TAK1 at Thr187, which is necessary for TAK1 kinase activity (Singhirunnusorn et al., 2005), and also enhanced the phosphorylation of p65 NF-κB at Ser536 (Fig. 6A). These changes coincided with a reduction in the levels of the inhibitor of κBα (IκBα), probably due to increased IκBα phosphorylation and subsequent degradation. Moreover, a time-course study showed a prolonged TAK1 phosphorylation in cells pretreated with BIRB 796 (Fig. 6B). Because okadaic acid, an inhibitor of serine/threonine phosphatases [especially protein phosphatase 2A, PP2A (Westermarck et al., 2001)] strongly increased TAK1 phosphorylation (Fig. 6B), we analyzed whether p38 inhibition might affect PP2A activity in this experimental system. As shown in Fig. 6C, p38 inhibition by BIRB 796 reduced PP2A activity in cells treated with T/I. Collectively, these results suggest that inhibition of p38 leads to the activation of the TAK1–NF-κB pathway, and that this effect might be mediated by inhibition of protein phosphatases, such as PP2A.

TAK1 and NF-κB mediate the effects of p38 inhibition on E-cadherin expression and on the acquisition of a spindle-like phenotype

To directly demonstrate the role of enhanced NF-κB signaling in the downregulation of E-cadherin induced by p38 inhibition, we

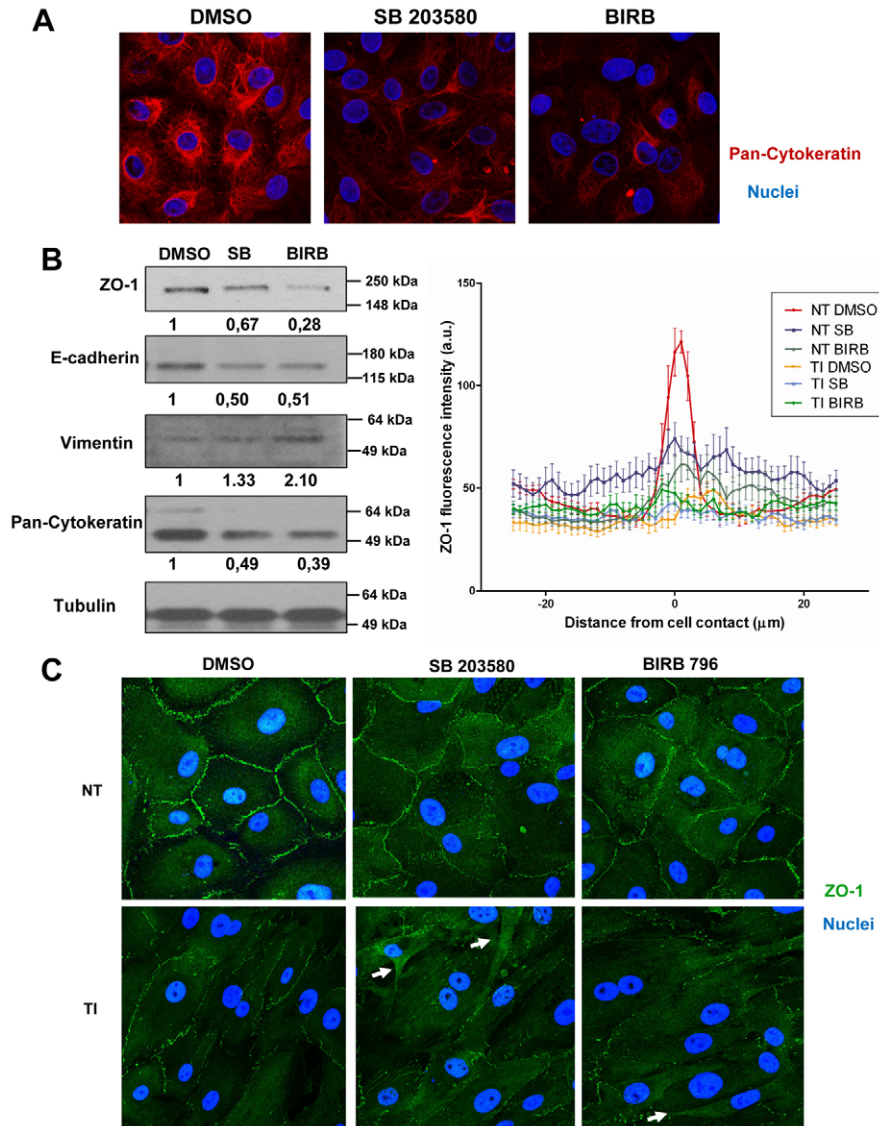


Fig. 3. p38 MAPK regulates the expression of EMT-associated molecules in MCs. (A) Effect of p38 inhibition on cytokeratin immunofluorescence. MCs were pretreated with 10 μ M SB203580, 250 nM BIRB 796 or DMSO for 56 hours before processing for immunofluorescence with a monoclonal antibody against pan-cytokeratin. Cell nuclei are shown in blue (Hoechst 33342). Representative confocal images are shown from one independent experiment of three performed. (B) Effect of p38 inhibition on ZO-1, vimentin and cytokeratin protein expression. MCs were treated with 10 μ M SB203580, 250 nM BIRB 796 or vehicle (DMSO) for 56 hours. Western blots show expression of ZO-1, E-cadherin, vimentin, pan-cytokeratin and γ -tubulin. A representative independent experiment is shown of three performed. (C) Effect of p38 inhibition on ZO-1 immunofluorescence. Bottom: MCs were treated with 10 μ M SB203580, 250 nM BIRB 796 or DMSO for 1 hour and then stimulated for 56 hours before processing for immunofluorescence with a polyclonal antibody against ZO-1. Cell nuclei are shown in blue (Hoechst 33342). White arrows show cells with fibroblastic shape devoid of ZO-1 at cell edges. Representative confocal images are shown from one independent experiment of three performed. Top: quantification of the results (see supplementary material Fig. S3C).

infected MCs with a retroviral vector encoding an I κ B α super-repressor, a non-degradable mutant form (Ser32Ala and Ser36Ala) of the repressor I κ B α . The I κ B α super-repressor rescued the enhanced downregulation of E-cadherin expression observed upon pharmacologic inhibition of p38 (Fig. 7A). Moreover, E-cadherin expression was also rescued by retroviral infection with TAK1 D175A, a catalytically inactive mutant of TAK1 that behaves as a dominant-negative mutant, and a similar result was obtained by pretreating MCs with a derivative of 5Z-7-oxoeanol, a highly specific and potent inhibitor of TAK1 catalytic activity (Yao et al., 2007), both for protein and mRNA expression (Fig. 7B,C). Interestingly, 5Z-7-oxoeanol inhibited the basal and cytokine-induced phosphorylation of p38 (Fig. 7B), supporting our hypothesis that p38 MAPK and the TAK1-NF- κ B activation pathway are linked by a negative feedback loop. Treatment with 5Z-7-oxoeanol also totally blocked the acquisition of a spindle-like phenotype, characteristic of EMT, upon cytokine stimulation in cells treated with p38 inhibitors (Fig. 8). These experiments demonstrate a causal link between p38 and NF- κ B, through the modulation of TAK1 activity, in the control of E-cadherin downregulation and EMT induction in MCs.

Discussion

This study aimed to characterize the role of p38 MAPK in the EMT undergone by primary mesothelial cells, in particular in the control of E-cadherin expression. Our results demonstrate that p38 maintains E-cadherin levels in MCs, acting as a break on cytokine-induced E-cadherin downregulation by modulating the TAK1-NF- κ B activation pathway. To our knowledge, this is the first study to show a role of p38 in supporting E-cadherin levels, and indicates a previously unknown role for p38 MAPK in impeding the progression of EMT. As demonstrated with different experimental approaches, inhibition of p38 activity led to downregulation of E-cadherin expression in MCs. RT-PCR experiments showed a parallel reduction of E-cadherin-encoding mRNA, suggesting that p38 acts in this experimental system at the level of mRNA expression. p38 inhibition was able to further reduce E-cadherin levels upon T/I treatment, which induces EMT in MCs (Yañez-Mo et al., 2003). We obtained opposite results by treating MCs with sodium arsenite, a known p38 activator. Hence, p38 activation counteracted E-cadherin downregulation and disappearance from the plasma membrane, as shown by western blot and confocal microscopy analysis.

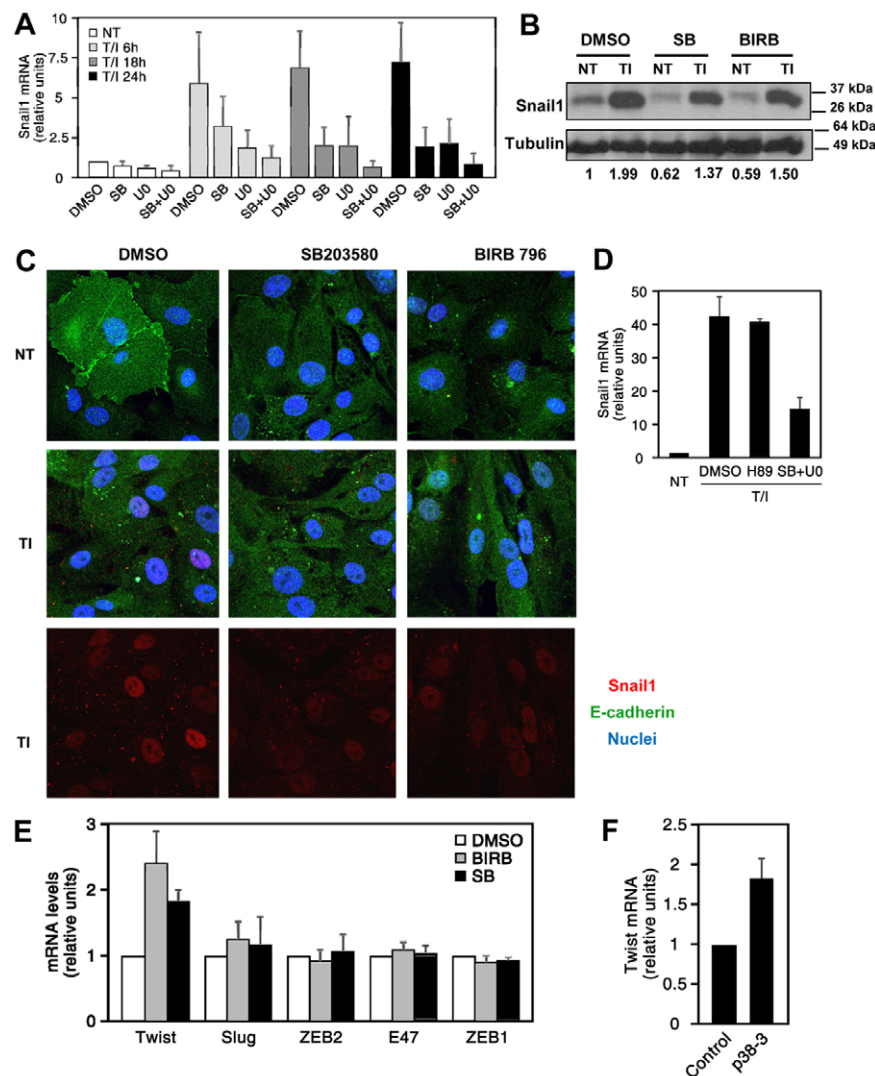


Fig. 4. p38 and ERK upregulate Snail1 expression during EMT by cytokine-stimulated MCs. (A) Inhibition of p38 or ERK reduces MC expression of Snail1. Cells were pretreated with 10 μ M SB203580 and 20 μ M U0126, alone or in combination, and then either not treated (NT) or stimulated for 6, 12 and 24 hours with T/I. Quantitative RT-PCR was performed on total RNA. Histone H3-encoding mRNA levels were used for normalization. Bars represent means \pm s.e.m. of duplicate determinations in three independent experiments. (B) Western blot showing the effect of p38 inhibition on Snail1 protein expression in MCs. Cells were pretreated with 10 μ M SB203580, 250 nM BIRB 796 or vehicle (DMSO) and either not treated (NT) or stimulated for 24 hours with T/I; γ -tubulin was detected as a loading control. (C) Effect of p38 inhibition on Snail1 immunofluorescence. MCs were treated as for B. Cells were fixed, permeabilized, stained with a mouse monoclonal antibody against Snail1 (red) and a rabbit monoclonal against E-cadherin (green) and subjected to confocal microscopy analysis. Panel shows immunofluorescence staining of Snail1 and E-cadherin expression. Cell nuclei are shown in blue (Hoechst 33342). Only Snail1 from T/I-stimulated cells is shown. The results shown are from a representative independent experiment of three performed. (D) Effect of MSK1/2 inhibition on Snail1 expression. MeT5A cells were pretreated with DMSO, 10 μ M H89 or SB203580 plus U0126 and stimulated for 12 hours with T/I as indicated. Quantitative RT-PCR was performed on total RNA, with Snail1-encoding mRNA amounts normalized to expression of histone H3-encoding mRNA. (E) Effect of pharmacologic p38 inhibition on the mRNA expression of the zinc finger transcription factors Twist, Slug, ZEB2, E47 and ZEB1 in MCs. Cells were pretreated as for B and mRNA expression determined by quantitative RT-PCR on total RNA, with normalization to histone H3-encoding mRNA levels. (F) Effect of p38 shRNA silencing on Twist expression. Cells were infected with lentiviral vector encoding a specific p38-targeting shRNA. Quantitative RT-PCR was performed on total RNA, with normalization to histone H3-encoding mRNA levels. Control cells were infected with nonspecific shRNA.

Treatment with p38 inhibitors also induced a disassembly of the cytokeleton filament network and a reduction of cytokeleton levels. This phenomenon was slower (56 hours) than E-cadherin downregulation (24–48 hours). p38 has been reported to phosphorylate cytokeleton at Ser73, this event allowing the formation of keratin granules, or Mallory bodies in the hepatocytes (Wöll et al., 2007). In MCs, cytokeleton disassembly and downregulation might favour the acquisition of a more motile phenotype, sustained by the increase of other intermediate filaments such as vimentin (Yañez-Mo et al., 2003). Also, prolonged treatment with p38 inhibitors reduced the expression and cellular localization of ZO-1. In particular, the appearance after treatment with T/I plus p38 inhibitors of cells with a fibroblastoid shape, which no longer localize ZO-1 at cell–cell junctions, suggests that an EMT process is taking place in these experimental conditions.

On the other hand, p38 inhibition increased the expression of vimentin, a mesenchymal marker. Vimentin has been reported to be phosphorylated upon p38 activation, and recent studies demonstrate that the p38–hsp27 pathway affects vimentin expression and filament assembly (Liu et al., 2010).

We focused our study on the control mechanism of E-cadherin transcription by p38. We analyzed the expression of transcriptional

factors relevant for E-cadherin downregulation, such as zinc finger proteins of Snail, ZEB and bHLH families (Peinado et al., 2007). We found that p38 inhibition limited the expression of Snail1, a factor that is rapidly induced by T/I treatment and plays a major role in the induction of EMT in MCs (Strippoli et al., 2008). With this respect, p38 inhibition is similar, albeit less strong, to the inhibition operated by ERK, which is a major Snail inducer. This result does not account for the role of p38 in controlling E-cadherin levels. Besides Snail, E-cadherin repression could be regulated by a wide array of factors. We analyzed the expression of other E-cadherin repressors and found that p38 inhibition leads to an increase of a bHLH factor, Twist, whereas other transcription factors such as ZEB1, ZEB2, E47 and Slug were not significantly affected by p38 inhibition. The expression of Twist upon p38 inhibition might account for the downregulation of E-cadherin during the induction of EMT in MCs. Interestingly, Twist has been reported to regulate Snail1 expression (Smit et al., 2009). In *Drosophila*, Twist is induced by Dorsal, a NF- κ B analog (Jiang et al., 1991). NF- κ B is an important mediator of inflammation, has been shown to play a role in a tumor model of EMT, is emerging as a central player in many models of EMT, and controls EMT in MCs (Huber et al., 2004; Strippoli et al., 2008). Similarly to E-

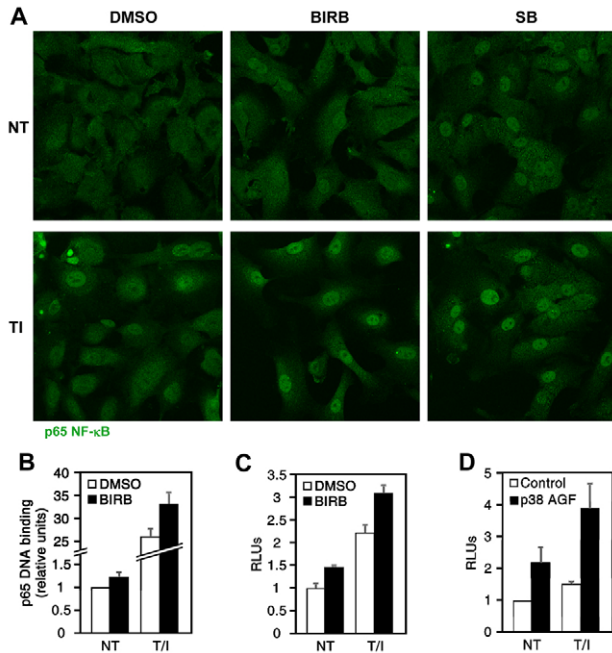


Fig. 5. Inhibition of p38 enhances NF- κ B nuclear translocation, DNA binding and transcriptional activity induced by T/I stimulation in MCs. (A) Confocal immunofluorescence analysis of NF- κ B expression and localization. MCs were pretreated for 1 hour with 10 μ M SB203580, 250 nM BIRB 796 or DMSO vehicle, and either not treated (NT) or stimulated for 2 hours with T/I. Fixed and permeabilized cells were stained with a polyclonal antibody against p65 NF- κ B. Images show the results of a representative independent experiment of three performed. (B) Effect of p38 inhibition on NF- κ B p65 DNA binding. MeT5A cells were pretreated for 1 hour with DMSO or 250 nM BIRB 796 and then stimulated as in A. After 2 hours, nuclear extracts were prepared and p65 DNA binding was assessed. Bars represent means \pm s.e.m. of four independent experiments. (C) Effect of pharmacologic inhibition of p38 on NF- κ B transcriptional activity. MeT-5A cells were transiently transfected with the KBF-luc reporter plasmid together with Renilla luciferase. Cells were pretreated with 250 nM BIRB 796 and stimulated for 9 hours as indicated. Bars represent means \pm s.e.m. of three independent experiments. (D) Effect of dominant-negative inhibition of p38 on NF- κ B transcriptional activity. MeT-5A cells were infected with Cop Green-tagged retrovirus encoding dominant-negative p38AGF or with empty virus (control). Cells were then transiently transfected with the KBF-luc reporter plasmid together with Renilla luciferase, followed by stimulation as indicated for 4.5 hours. Bars represent means \pm s.e.m. of triplicate determinations in three independent experiments.

cadherin, NF- κ B has also been demonstrated to inhibit cytokeratin expression in mammary epithelial cells (Chua et al., 2007). Thus, we hypothesize that p38 might control E-cadherin and cytokeratin levels through its effect on NF- κ B activity.

We investigated the relationship between p38 and NF- κ B in our experimental system. We found that p38 inhibition led to increased NF- κ B nuclear translocation, binding to specific DNA sequences and transcriptional activity. These effects were seen both at basal levels and in cells stimulated with T/I. Depending on the stimuli and the experimental models, p38 can both activate or inhibit NF- κ B transcriptional activity. Various stimuli known to induce p38 activation, such as salicylate, sorbitol, arsenite and UV radiation, can induce NF- κ B inhibition (Orr et al., 2005; Ivanov, 2000).

Interestingly, Twist plays a role in a major negative feedback for NF- κ B activity. *Twist2*^{-/-} mice show uncontrolled inflammation

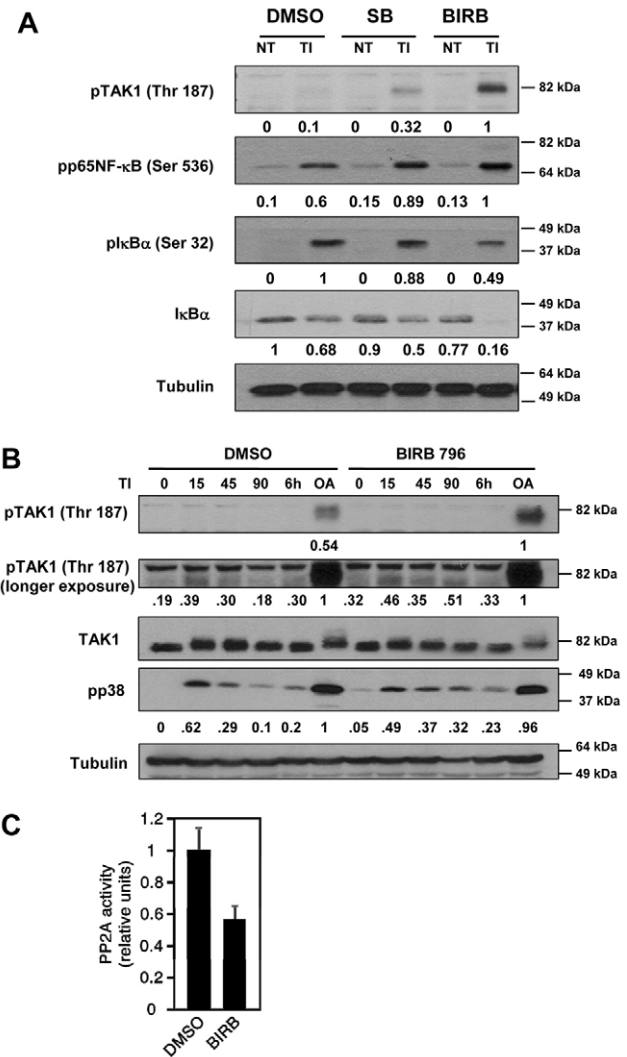
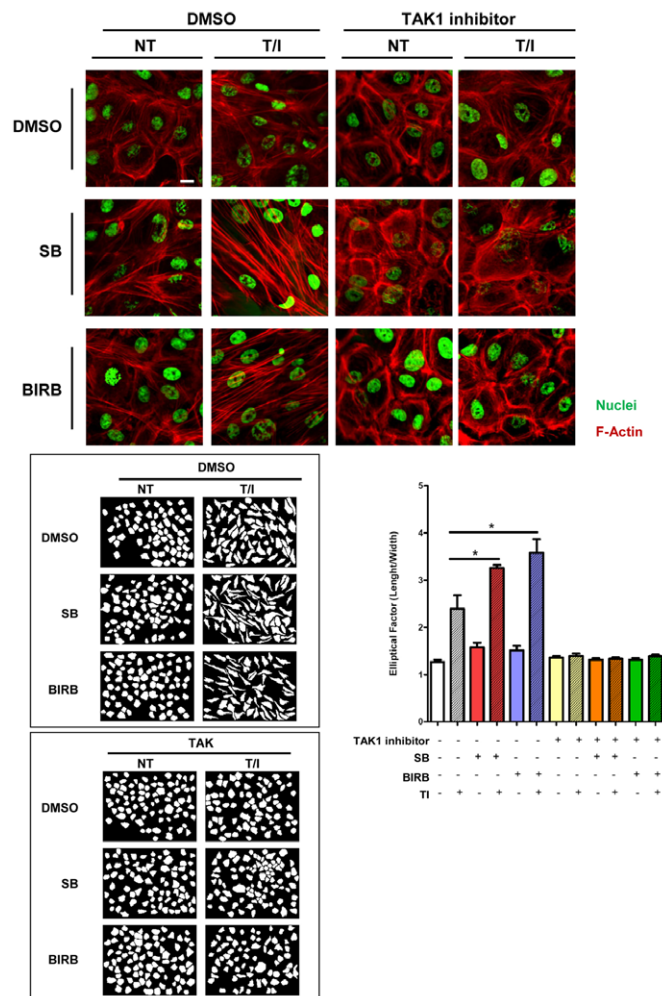
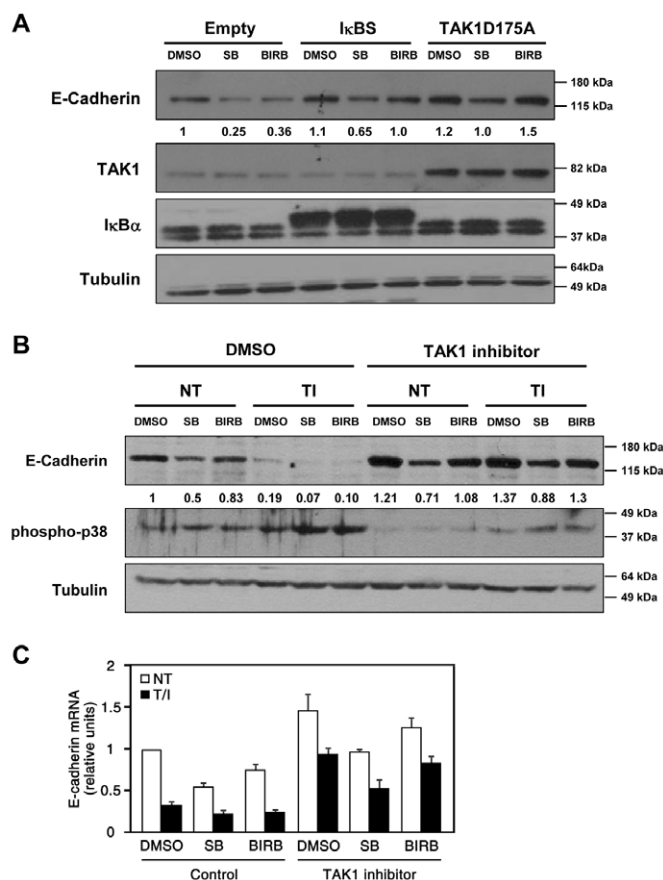


Fig. 6. p38 inhibition increases the activity of the TAK1-NF- κ B activation pathway induced by T/I stimulation in MCs, while decreasing PP2A activity. (A) Western blots showing the levels of phospho-TAK1 (Thr187), phospho-NF- κ B (Ser536), phospho-I κ B α (Ser32), and total I κ B α expression in MC lysates. MCs were pretreated with 10 μ M SB203580, 250 nM BIRB 796 or DMSO vehicle and either not treated (NT) or stimulated for 30 minutes with T/I. Expression of γ -tubulin was detected as a loading control. The results shown are from a representative independent experiment of three performed. (B) Western blots showing the levels of phospho-TAK1 (Thr187) in cells stimulated for 15 minutes with T/I. When indicated, cells were pretreated for 15 minutes with okadaic acid (OA) – following DMSO or BIRB 796 treatment – and then stimulated for 15 minutes with T/I. The results shown are from a representative independent experiment of three performed. (C) Phosphatase assay of MCs pretreated for 2 hours with DMSO or 250 nM BIRB 796. Cells were then stimulated for 30 minutes with T/I. Cells were then lysed in phosphatase buffer and immunoprecipitated with a polyclonal antibody against PP2A. Phosphatase assays were executed according to the instructions of the manufacturer. Bars represent means \pm s.e.m. of three independent experiments.

due to aberrant NF- κ B activity (Sosic et al., 2003). We therefore studied how p38 inhibition might lead to increased NF- κ B activity. We focused on TAK1, a MAPK kinase rapidly induced by both IL-1 β and TGF- β 1 and playing a role in the activation of p38, JNK and NF- κ B signaling (Shim et al., 2005). Pretreatment with



p38 inhibitors increased TAK1 phosphorylation at Thr187 induced by T/I. This event correlated with increased phosphorylation of NF-κB at Ser536, which is linked to NF-κB activation, and with increased IκBα phosphorylation and degradation. All these events might account for increased NF-κB activity in cells pretreated with p38 inhibitors. TAK1 controls the NF-κB pathway upon TNFα stimulation by activating the high-molecular-weight IκB kinase (IKK) complex, which phosphorylates IκBα at Ser32 and Ser36 and NF-κB at Ser536 (Sakurai et al., 2003). Moreover, TAK1 has

been recently demonstrated to mediate TGF-β1-induced NF-κB effects in a tumor model of EMT (Neil and Schiemann, 2008). Our results thus suggest that the effects of p38 inhibition on NF-κB are related to increased TAK1 activity. Because TAK1 phosphorylation is increased upon treatment with okadaic acid, a serine/threonine phosphatase inhibitor, we hypothesized that p38 might regulate TAK1 activity via this route. In agreement with this hypothesis, we found that treatment with BIRB 796 markedly inhibits PP2A activity in MCs stimulated with T/I. It has already been demonstrated in other experimental settings that p38 co-immunoprecipitates with PP2A and regulates its activity in response to diverse stimuli, including TNFα and sodium arsenite (Grethe et

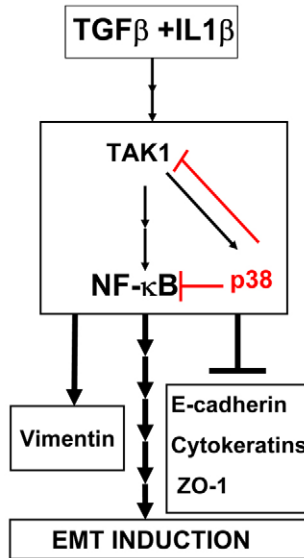


Fig. 9. Role of p38 in the regulation of E-cadherin expression and EMT through its action on the TAK1–NF- κ B pathway. See Discussion for details.

al., 2006; Avdi et al., 2002; Westermarck et al., 2001). Moreover, PP2A is stably associated with TAK1 and negatively regulates its activation by TGF- β 1 (Kim, S. I. et al., 2008). Alternatively, p38 might affect TAK1 activity through the phosphorylation of the TAK1 subunit TAB1, as proposed by Cheung and co-workers (Cheung et al., 2003).

Our analysis of TAK1–NF- κ B signaling showed that inhibition of the TAK1–NF- κ B pathway rescues the downregulation of E-cadherin induced by p38 inhibition. These results are summarized in the model shown in Fig. 9. TAK1 and NF- κ B play a major role, according to our results, in mediating the effect of p38 on E-cadherin expression; however, we cannot exclude TAK1–NF- κ B-independent effects of p38 on E-cadherin in our experimental system. In this regard, TAK1 would activate p38, which in turn would trigger a negative feedback loop on the TAK1–NF- κ B pathway to control excessive EMT.

Our results show that p38 promotes E-cadherin expression in MCs both under basal conditions and upon cytokine treatment. However, the difference between basal and cytokine-stimulated conditions in this experimental system might be simply a quantitative matter because MCs constitutively produce low levels of TGF- β 1 (Loureiro et al., 2010) that might favor the transition towards a mesenchymal phenotype. Because the decision between epithelial and mesenchymal fate is the outcome of a balance of numerous extracellular signals (Kalluri and Weinberg, 2009), we hypothesize that basal p38 activity in untreated MCs might contribute to the maintenance of the epithelial state, helping to prevent excessive transdifferentiation when the epithelium is exposed to inflammatory stimuli.

In this regard, unlike ERK, p38 activity is increased in confluent cultures with respect to dispersed cells (Houde et al., 2001; Faust et al., 2005); this is a particularly interesting finding because cell scattering is characteristic of EMT. We confirmed that p38 is activated in MCs under resting conditions, and that its activation increases with confluency. p38-mediated inhibition of EMT induction might reflect findings in other experimental systems, which show that p38 controls cell differentiation and might

contribute to proliferation arrest and tumor suppression (Houde et al., 2001; Perdiguero et al., 2007; Wagner and Nebreda, 2009). Moreover, a recent study identifies p38 at a crossroad of pro-inflammatory and anti-inflammatory responses (Kim, C. et al., 2008). On the other hand, p38 activity induced by pro-inflammatory stimuli, concomitant with other pathways (such as JNK and ERK), might be responsible for the effects linked to chronic inflammation and EMT, such as increased cell motility and invasion or extracellular matrix protein production, as demonstrated in other experimental systems (Bakin et al., 2002; Bhowmick et al., 2001). In contrast to our results, recent studies (Zohn et al., 2006; Liu, Y. et al., 2008) reported a downregulation of E-cadherin caused by p38 activation during mouse gastrulation and in lung morphogenesis. Interestingly, in these and other studies, mainly performed in tumoral or developmental models, E-cadherin is not regulated by p38 at a transcriptional level. These discrepancies with our results can be explained by the heterogeneity of different experimental approaches and EMT models.

Overall, a deeper understanding of the role of p38 in a chronic inflammatory model (such as the EMT of MCs) might be useful to better understand the role of this kinase in inflammation and other pathophysiological systems, in order to design more efficacious and selective anti-inflammatory strategies.

Materials and Methods

Isolation and culture of mesothelial cells

Human mesothelial cells (MCs) were obtained by digestion of omentum samples from patients undergoing unrelated abdominal surgery (Stylianou et al., 1990). Samples were digested in 0.125% trypsin containing 0.01% EDTA. Cells were cultured in Earle's M199 medium supplemented with 20% fetal calf serum, 50 U/ml penicillin, 50 μ g/ml streptomycin, and 2% Biogro-2 (containing insulin, transferrin, ethanolamine and putrescine) (Biological Industries, Beit Haemek, Israel). To induce EMT, MCs were treated with a combination of human recombinant TGF- β 1 (0.5 ng/ml) and IL-1 β (2 ng/ml) (R&D Systems, Minneapolis, MN) as described previously (Yanez-Mo et al., 2003). Although TGF- β 1 and IL-1 β both induce EMT phenotypic changes when administered separately, combined stimulation is necessary to induce genuine EMT. The cytokine doses used were within the range of those detected in peritoneal dialysis fluids from peritonitis patients (Lai et al., 2000) and are similar to those used in previous studies (Yanez-Mo et al., 2003; Yang et al., 1999). The human mesothelial cell line MeT-5A (ATCC, Rockville, MD) was cultured in Earle's M199 medium as above and stimulated with the same doses of TGF- β 1 and IL-1 β .

Antibodies and chemicals

Monoclonal antibodies against E-cadherin were purchased from BD (Becton-Dickinson Laboratories, Mountain View, CA) and Cell Signaling (Danvers, MA); monoclonal antibodies against tubulin, pan-cytokeratin and vimentin were from Sigma (St Louis, MO); polyclonal antibodies against p38, phospho-p38, ERK and phospho-ERK, TAK1, phospho-TAK1 and phospho-I κ B α were from Cell Signaling; polyclonal antibody against ZO-1 was from Zymed (South San Francisco, CA); polyclonal antibodies against p65 NF- κ B, I κ B α were from Santa Cruz Biotechnology (Santa Cruz, CA); monoclonal anti-PP2Ac (PP2A catalytic subunit) was from Upstate (Millipore, Temecula, CA). The anti-Snail1 monoclonal antibody was a kind gift from Antonio Garcia de Herreros, IMIM, Barcelona, Spain (Franci et al., 2006). Okadaic acid was from Sigma; U0126 was from Calbiochem (EMD, Darmstadt, Germany); SB203580 was from Assay Designs (Ann Arbor, MI); BIRB-796 was from Phil Cohen's laboratory (University of Dundee, UK). 9-Epimer-11,12-dihydro-(5Z)-7-oxozeanol was from AnalytiCon Discovery (NP-00924), Potsdam, Germany. Alexa Fluor 647–phalloidin was purchased from Invitrogen (Carlsbad, CA).

Western blotting and immunoprecipitation

MC monolayers were lysed in modified RIPA buffer [50 mM Tris-HCl pH 7.4, 1% NP-40, 0.1% SDS, 0.25% Na-deoxycholate, 150 mM NaCl, 1 mM EDTA, 1 mM EGTA, 1 mM PMSF, 1 μ g/ml each of aprotinin, leupeptin and pepstatin, and 25 mM NaF (all from Sigma)]. For assays of Snail1 protein expression, cells were lysed in buffer containing 50 mM Tris-HCl pH 7.4, 1% Triton X, 0.2% SDS, 1% Na-deoxycholate, 150 mM NaCl, 1 mM EDTA, 1 mM EGTA, 1 mM PMSF, 1 μ g/ml each of aprotinin, leupeptin and pepstatin, 25 mM NaF, 1 mM sodium orthovanadate and 2 mM betaglycerophosphate. Total cell extracts (700 μ g of protein) were immunoprecipitated using monoclonal antibody anti-PP2Ac and anti-mouse agarose. Immunoprecipitates were washed with lysis buffer three times. Then, they were washed with the phosphatase lysis buffer to measure PP2A phosphatase activity.

Equal amounts of protein were resolved by SDS-PAGE. Proteins were transferred to nitrocellulose membranes (Amersham Life Sciences, Little Chalfont, UK) and probed with antibodies using standard procedures. Nitrocellulose-bound antibodies were detected by chemiluminescence with ECL (Amersham Life Sciences).

Confocal microscopy and immunofluorescence

Cells were fixed for 20 minutes in 3% formaldehyde in PBS, permeabilized in PBS containing 0.2% Triton X-100 for 5 minutes, and blocked with 2% BSA for 20 minutes. For E-cadherin staining, cells were fixed and permeabilized in cold methanol for 5 minutes. Secondary antibodies (conjugated to Alexa Fluor 647, Alexa Fluor 488 and Alexa Fluor 541) and Hoechst 33342 were from Pierce Chemical (Rockford, IL). Confocal images were acquired with a Leica SP5 Confocal Microscope. The elliptical factor (length/width) of cells as a measure of elongation was determined by using the MetaMorph software (Universal Imaging). For the membrane and cytoplasm measurements, fluorescence intensity profiles were analyzed with Leica LAS-AF software and represented graphically with Prism Graph-Pad 5.0.

Infection of MeT5A cells and MCs with retroviral and lentiviral vectors

MCs were infected with pRV-IRES-CopGreen retroviral vectors (Genetrix, Madrid, Spain) encoding p38AGF, a dominant-negative mutant of p38; WT p38. MCs were infected with a super-repressor I κ B α mutant harboring mutations Ser32Ala and Ser36Ala. The I κ B α super-repressor blocks NF- κ B nuclear translocation induced by TGF- β 1 and IL-1 β , whereas empty vector has no effect (Strippoli et al., 2008). MCs were also infected with a catalytically inactive TAK1 mutant (Asp175Ala, from Phil Cohen's laboratory, University of Dundee, Scotland); or with empty pRV-IRES-CopGreen vector as control. Twenty-four hours before infection, MCs were seeded into six-well plates (2×10^5 cells per well), and retrovirus-producing 293T cells were seeded at 3×10^6 cells per 10-cm plate. For infection, 293T cell supernatants were filtered through a 0.45- μ m filter (Whatman, Dassel, Germany), and 5 μ g/ml polybrene (Sigma-Aldrich, St Louis, Missouri) was added to the filtrate. Thereafter, medium was removed from the MCs and replaced with 293T cell supernatants containing the retrovirus. This process was repeated twice at 24-hour intervals. Twenty-four hours after the last exposure to retrovirus, infection efficiency was monitored by fluorescence microscopy (Carl Zeiss, Standort Göttingen, Germany) or FACS analysis (BD FACS Canto, Becton-Dickinson Laboratories, Mountain View, CA). Alternatively, MCs were infected with virus encoding shRNA specific for p38 α , which was a kind gift from Angel Nebreda, CNIO, Madrid, Spain. MC infection was performed according to the manufacturer's protocols.

RNA interference

MCs were grown on 48-well plates (4×10^4 cells per well) for 24 hours and transfected twice with a 72-hour interval using Dharmafect 1 (0.8 μ l per well) and the ON-TARGETplus SMARTpool directed against human Twist1 (60 pmol per well) or a nonspecific control siRNA (Dharmacon, Lafayette, CO). At 24 hours after the last transfection, cells were treated as indicated and RNA was extracted 48 hours later. Knockdown efficiency and E-cadherin levels were determined by quantitative PCR (see above).

NF- κ B p65 DNA binding assay

MeT5A cells were grown to confluence in 10-cm dishes and treated as indicated. Cells were scrapped in 1 ml of cold complete medium, pelleted and washed with cold PBS. Nuclear extracts were prepared using the NE-PER nuclear and cytoplasmic extraction reagents (Thermo Scientific, Rockford, IL) and protein concentration determined with the Bio-Rad protein assay (Bio-Rad Laboratories, Munich, Germany). Nuclear extracts (7 μ g) were used to determine p65 DNA binding activity with the NF- κ B p65 transcription factor kit (Thermo Scientific) according to the manufacturer's instructions. The chemiluminiscent signal was measured with the GloMax-Multi Microplate Multimode Reader (Promega).

PP2A phosphatase activity

PP2A activity was determined in PP2A immunoprecipitates using a serine/threonine phosphatase (PPase) assay system (Promega V2460). PP2A immunocomplexes were washed with a phosphatase lysis buffer (20 mM HEPES pH 7.4, 10% glycerol, 0.1% NP-40, 30 mM β -mercaptoethanol, 1 mM EGTA) and PPase activity measured following the manufacturer's protocol using a PP2A-specific buffer (50 mM imidazole pH 7.2, 0.2 mM EGTA, 0.03% β -mercaptoethanol, 0.1 mg/ml BSA) for the assay. Free phosphate generated from a phospho-peptide was quantified by measuring the absorbance (600 nm) of a molybdate/malachite green/phosphate complex.

Cell transfection and luciferase assays

NF- κ B transcriptional activity was measured by transient transfection of MeT5A cells with the KBF-luc reporter plasmid and subsequent luciferase activity assay (Castellanos et al., 1997). Briefly, 2×10^5 cells were transfected with 2 μ g of the KBF-luc reporter plasmid together with 500 ng of the reporter plasmid pRL-null, which bears a promoterless Renilla Luciferase gene (Promega, Madison, WI). Transfections were performed by incubating cells for 4 hours with a mixture of DNA and Lipofectamine 2000 at a ratio of 1:2.5 (Invitrogen, Carlsbad, CA) in serum-free medium. After transfection, cells were pretreated overnight with vehicle (DMSO) or U0126 (20 μ M). Cells were then stimulated with T/I for the times indicated.

Luciferase activity was measured with the Dual-Luciferase Reporter Assay System (Promega) according to the manufacturer's instructions and determined in a Sirius Single Tube Luminometer (Berthold Detection Systems, Pforzheim, Germany). All experiments were performed in triplicate.

Reverse-transcriptase polymerase chain reaction

Total RNA was extracted with the RNeasy kit (Qiagen, Hilden, Germany), and complementary DNA was obtained from 500 ng total RNA with an Omniscript RT kit (Qiagen). Quantitative PCR was conducted in a LightCycler (Roche Diagnostics, Mannheim, Germany) using a SYBR Green kit (Roche Diagnostics). The following specific primer sets were used: E-cadherin, 5'-TGAAGGTGACAGAGCCTCTG-3' and 5'-TGGGTGAATTCGGGCTTGTT-3'; Snail1, 5'-GCAAATACTGCAA-CAAGG-3' and 5'-GCACCTGGTACTTCTTGACA-3'; E47, 5'-AGATGCACCT-CAAGTCGGAC-3' and 5'-TCTCCAACCACACCTGACAC-3'; ZEB1, 5'-GGTG-GCATACACTACTCAAC-3' and 5'-CAGAGTCATTCTGATCCTCTAC-3'; ZEB2, 5'-CGCTTGACATCACTGAAGGA-3' and 5'-CTTGCCACACTCTGTGCATT-3'; Slug, 5'-CTTTTCTTGCCTCACTGC-3' and 5'-AGCAGCCAGATTCTCATGT-3'; Twist, 5'-GGAGTCCGCAGTCTTACGAG-3' and 5'-TCTGGAGACCTGGTA-GAGG-3'; histone H3 (for normalization), 5'-AAAGCCGCTCGAAGAGTGGC-3' and 5'-ACTTGCCCTCTGCAAAGCAC-3'. After amplification, PCR product identity was confirmed by melting-curve analysis and gel electrophoresis.

Statistical analysis

Statistical significance was determined with a Student's *t*-test (unpaired *t*-test) that was performed using Graph pad 4.0 software, where *P* values of <0.05 were considered significant.

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Supplementary material available online at

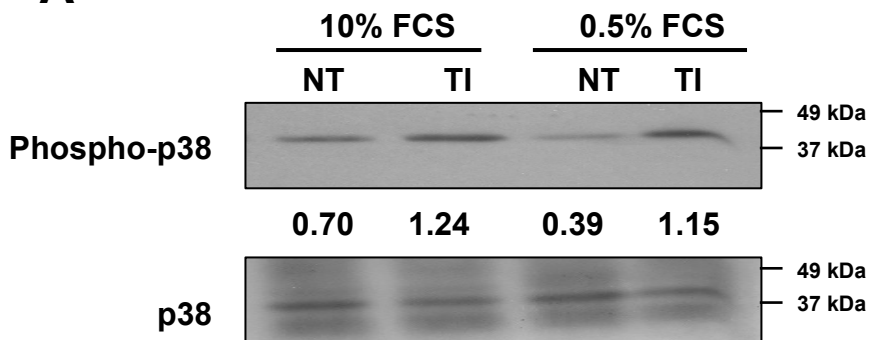
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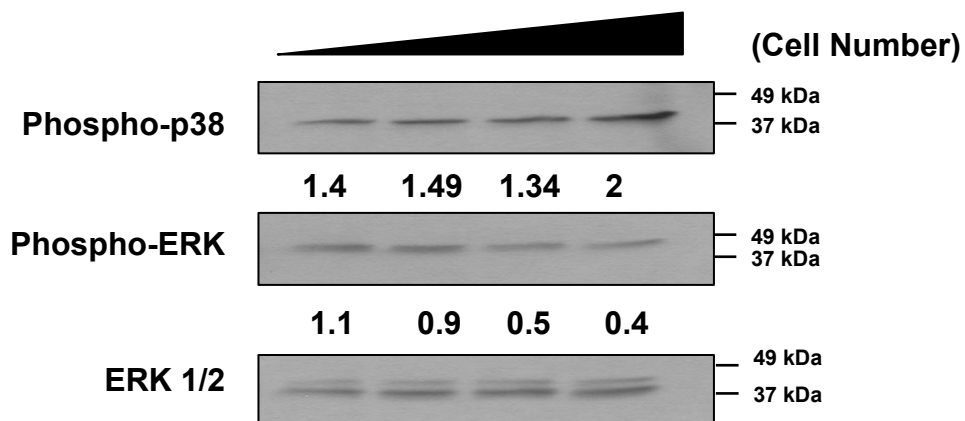
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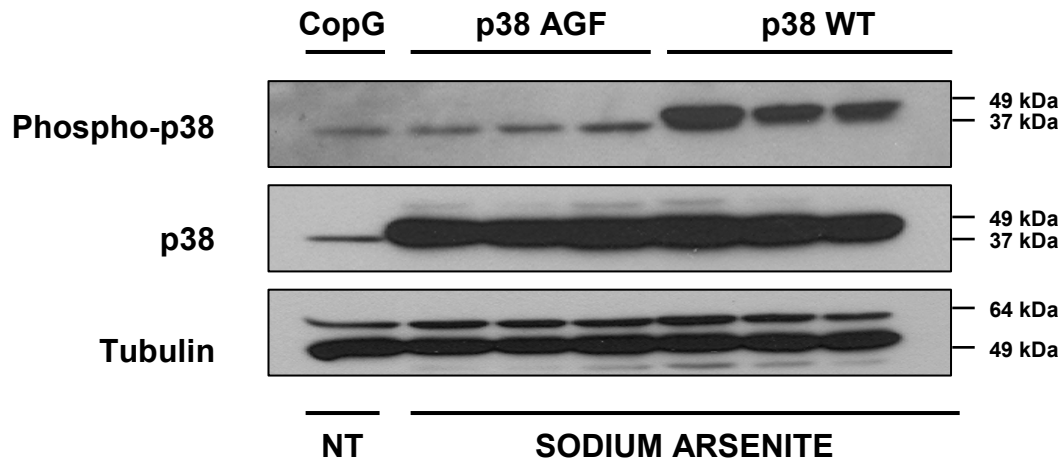
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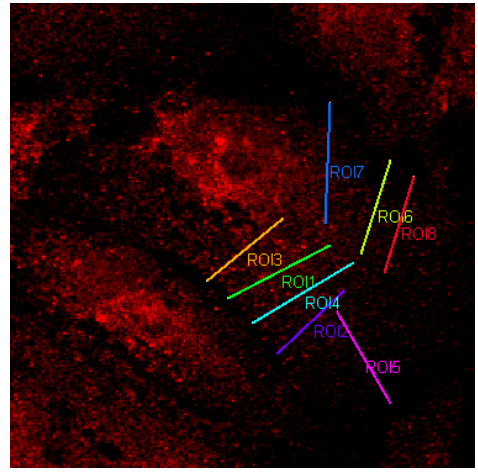
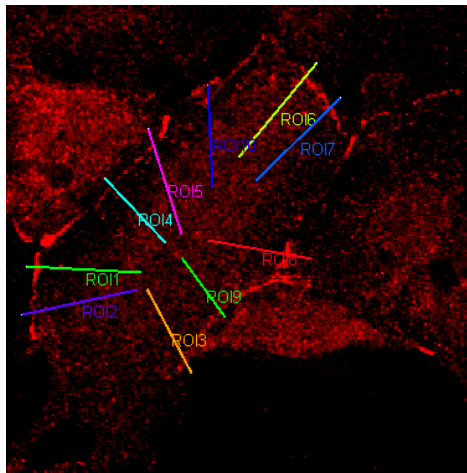
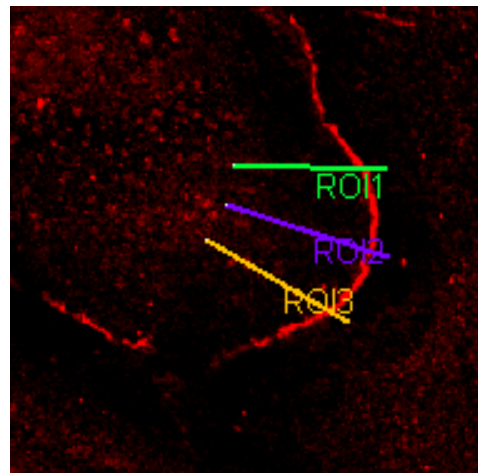
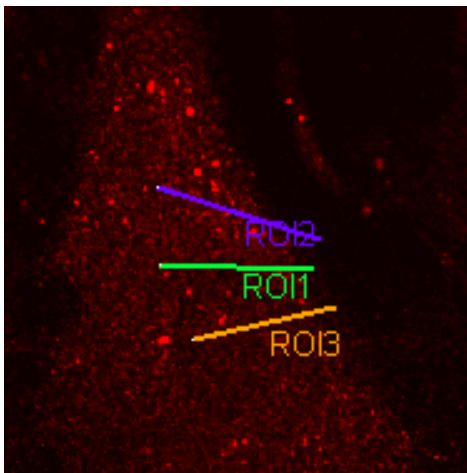
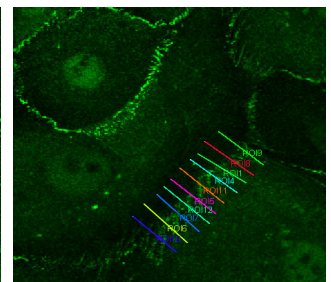
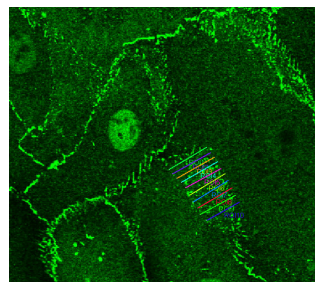
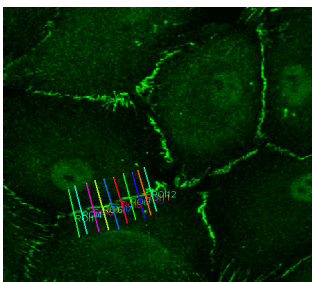
A



B





A**Scrambled shRNA****p38-1 shRNA****E-cadherin****B****TI****TI + Ars 25****E-cadherin****C****DMSO****SB****BIRB****NT****ZO-1****T/I**