CHAPTER VI

STATHMIN MODULATES TGFβ-MEDIATED EMT FORMATION IN PROSTATE CANCER CELLS BY ACTIVATING p38MAPK

EMT, which entails a transformation of the polarized epithelial cells to a highly motile mesenchymal phenotype, is a central process during various cellular processes such as embryonic development, chronic inflammation and cancer progression. In recent years, the role of EMT in cancer progression and metastasis have been studied with increasing intensity both *in vitro* using tissue culture models and *in vivo* in transgenic mouse tumor models. Compelling evidence implicates EMT in promoting invasion and metastasis in a variety of human malignancies. However, the role of EMT in PCa progression towards a more invasive phenotype remains poorly studied. We have established a novel primary cell culture model to study the emergence of EMT in human PCa. However, since the life span of primary HPE and EMT cultures are limited to 5 to 8 passages, the primary cell culture model is restricted in its scope for functional analyses of molecular networks. Hence, a well-defined tissue culture model will contribute to elucidating molecular events during emergence of EMT in PCa and can lead to novel diagnostic and therapeutic strategies.

DU145 cells as a model to study stathmin-regulated EMT phenotype

A number of cell lines including LNCaP, PC-3, DU145 and BPH were tested to determine which line could be utilized to analyze the role of stathmin in EMT. It has been well documented that TGFβ signaling in association with Ras or Receptor Tyrosine

Kinases (RTKs) causes EMT and enhances the invasive potential of cells in both *in vitro* and *in vivo* models. Hence, we treated PC-3, LNCaP, DU145 and BPH with 5.0 ng/ml of TGFβ or 10.0ng/ml EGF for 7 days alone or in combination (Fig. 22).

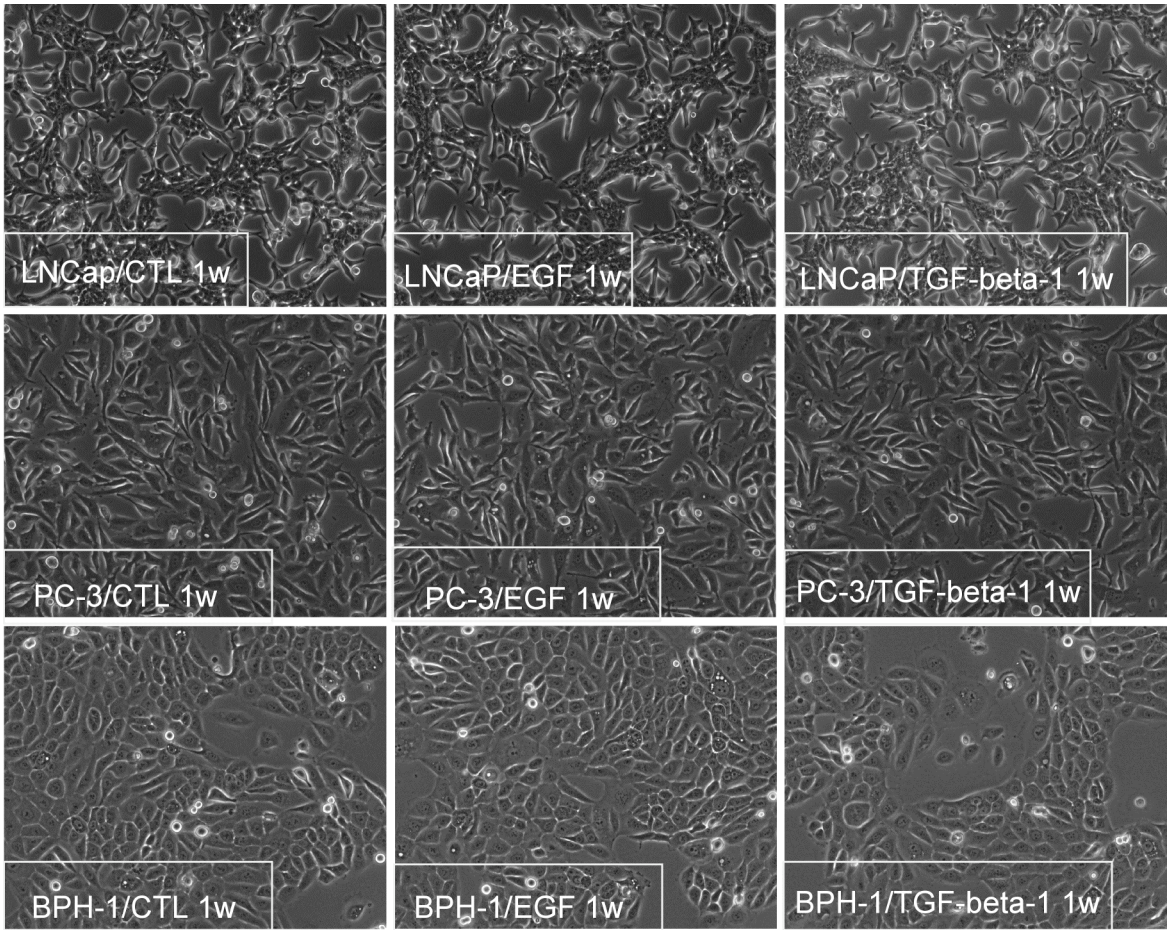

Figure 22. TGFβ induced EMT in PCa cells. LNCaP, PC-3 and BPH cells were treated with 5ng/ml and 10ng/ml TGFβ and EGF respectively, alone or in combination for 7 days. EMT was not observed in any of these cell lines.

Transition to typical spindle-shaped EMT cells is not observed in LNCaP, PC3 or BPH cells (Fig. 22). However, DU145 cells undergo EMT in response to both TGF-β1 and EGF treatment (Fig. 23 Panel A). TGF-β1 promotes EMT in 100% of cells, whereas EGF alone is less effective, inducing EMT in <20% of cells(Fig. 23 Panel A). EMT is initiated on Day 3 of treatment and completed on Day 7 of treatment with all cells becoming

spindle shaped (Fig. 23 Panel B). The emergence of EMT-like cells were accompanied by decreased E-cadherin and a simultaneous increase in vimentin (Fig. 23 Panel C). Interestingly, stathmin is dramatically downregulated in the DU145 cells after 7 days of TGFβ treatment (Fig. 23 Panel D) i.e. precisely at the time when DU145 cells transform into EMT cells. However, stathmin downregulation is not observed in response to EGF treatment (Fig. 23 Panel D). This confirms our finding, in the primary cell culture, that downregulation of stathmin coincides with emergence of EMT cells. Furthermore, this establishes the DU145 cells as a suitable model system to elucidate the molecular events regulated by stathmin in the EMT phenotype.

We sought to elucidate the mechanism by which TGFβ regulates stathmin expression. Since, it took seven days of chronic TGFβ treatment before stathmin expression was downregulated, it appears unlikely that TGFβ exerts a transcriptional control over stathmin expression. Hence, we hypothesized that TGFβ compromises the stability of stathmin through a yet unknown mechanism. DU145 cells were treated with cycloheximide (Chx) in presence or absence of TGFβ (Fig. 23 Panel E) for up to 72 hours. Chx treatment prevents protein synthesis and thus enables us to measure the stability of proteins that had already been translated before Chx treatment. Western blot and densitometric analysis of stathmin expression reveals that TGFβ treatment in presence of Chx leads to ~1.5-fold reduction in stathmin expression compared to when treated with Chx alone (Fig. 23 Panel E and F). Thus chronic TGFβ treatment destabilizes stathmin resulting in its downregulation.

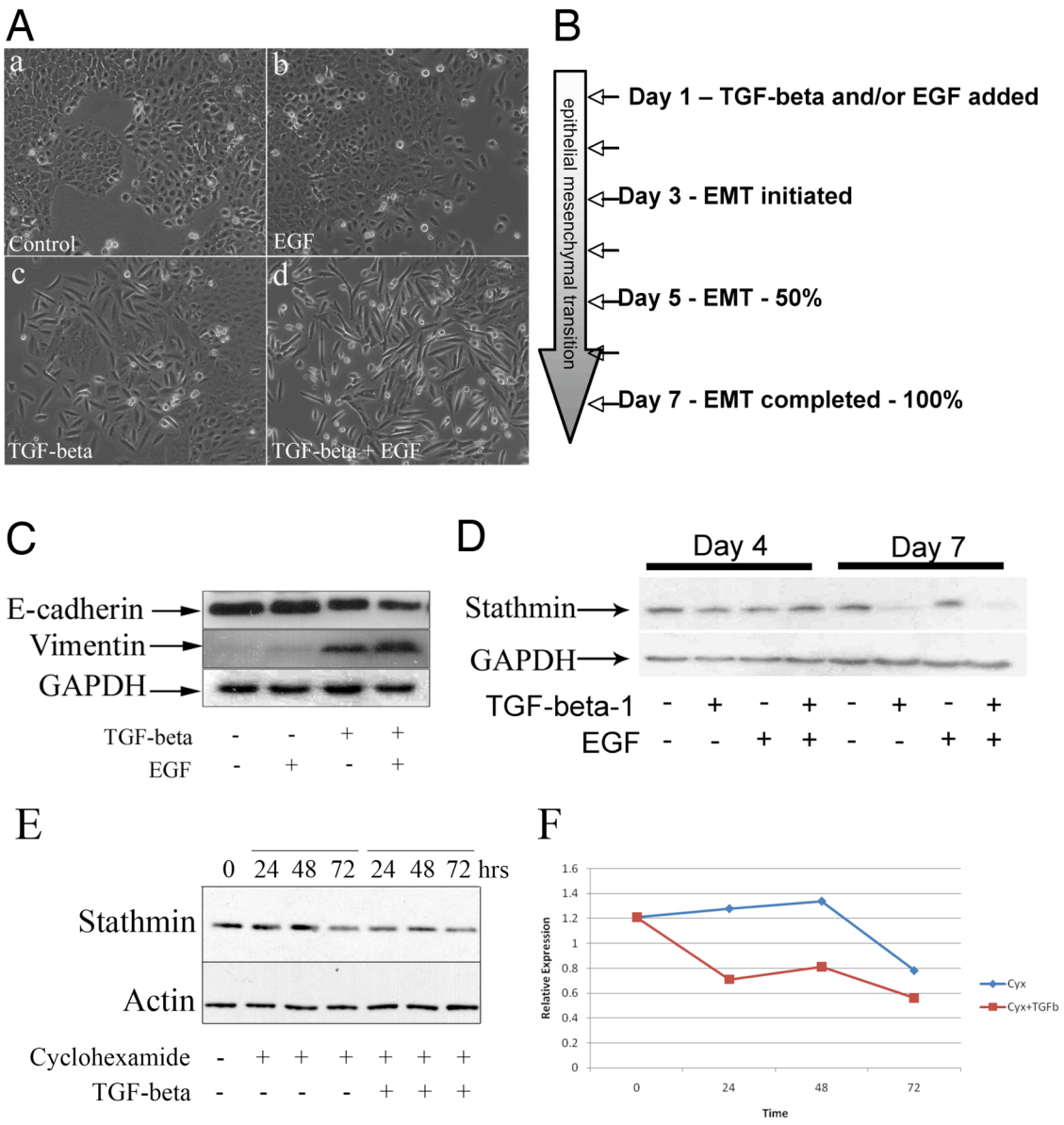

Figure 23. TGFβ induces EMT and destabilizes stathmin in DU145 cells. A. EMT is induced in DU145 cells following 7 days of TGFβ treatment. EGF by itself does not produce EMT. B. Timeline of EMT phenotype in DU145 cells. C. E-cadherin is downregulated and Vimentin is upregulated in DU145 cells treated with TGFβ. D. Stathmin is downregulated following chronic TGFβ treatment. EGF has no effect on stathmin expression. E. Stathmin is destabilized by TGFβ treatment. DU145 cells were treated with Chx and TGFβ alone or in combination for 72 hours. Stathmin expression was analyzed by western blot analysis. F. Densitometric analysis to demonstrate TGFβ treatment destabilizes stathmin.

Stathmin regulates TGFβ-mediated EMT in DU145 cells

We next sought to elucidate more directly the role of stathmin in EMT phenotype. We reasoned that since stathmin downregulation coincides with emergence of EMT in our model, knockdown of stathmin in DU145 cells could hasten the appearance of EMT in response to TGFβ treatment. We used SmartPool siRNA against stathmin to knock down its expression in DU145 cells. Indeed, when stathmin is knocked down, EMT cells appear spontaneously within 1 day and without TGFβ treatment (Fig. 24).

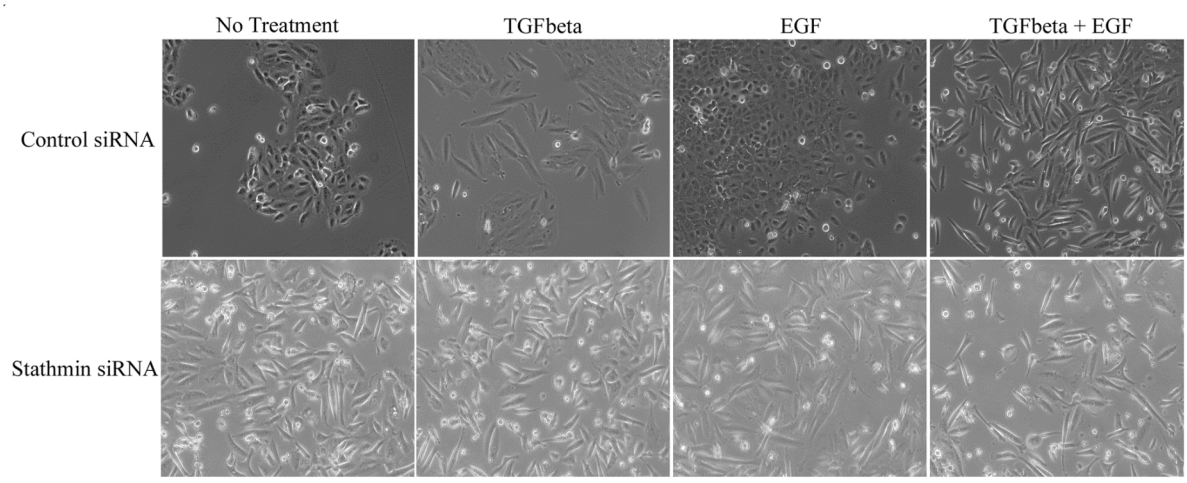

Figure 24. RNAi mediated downregulation of stathmin induces EMT in DU145 cells without TGFβ treatment. SMARTpool siRNA was used to knock down stathmin. Cells transfected with non-targeting control siRNA, requires 7 days of TGFβ treatment to elicit EMT phenotype. However, cells transfected with stathmin siRNA exhibit EMT within 24 hours and without TGFβ treatment.

In contrast, cells transfected with non-targeting control siRNA require 7 days of TGF-β1 treatment to undergo EMT (Fig 24). Immunnocytochemical (Fig. 25 Panel A) and western blot analysis (Fig. 25 Panel B) exhibits that knockdown of stathmin resulted in increased expression of mesenchymal marker vimentin and a concomitant decrease in epithelial

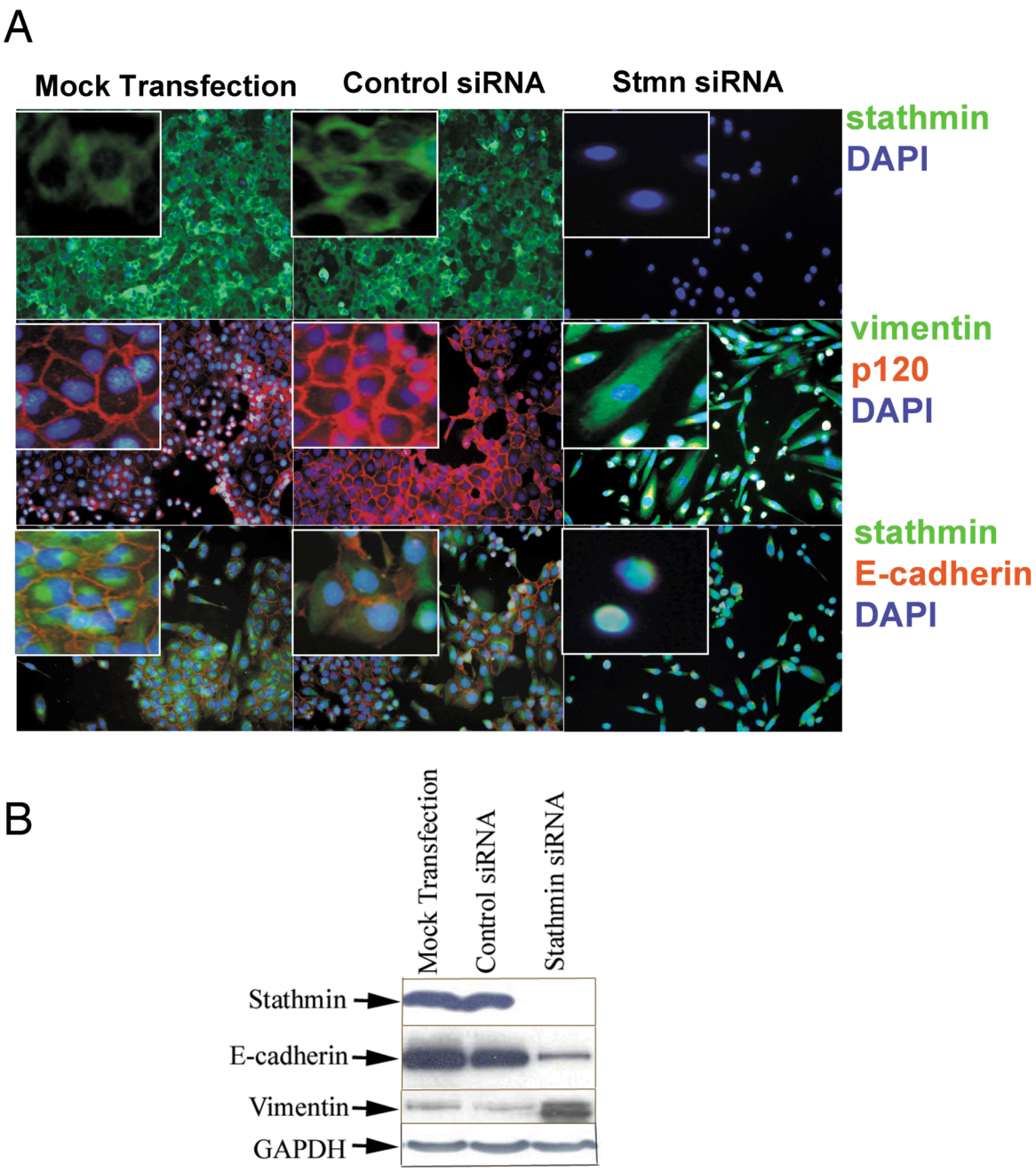

Figure 25. Stathmin downregulation in DU145 cells results in EMT-like cells with decreased E-cadherin and increased vimentin expression. A. Immunofluorescence analysis reveals decreased E-cadherin and p120 and increased vimentin expression in DU145 cells transfected with stathmin siRNA. Mock transfection and non-targeting control siRNA transfection does not affect E-cadhein, p120 and Vimentin expression. B. Western blot analysis to confirm stathmin downregulation in cells transfected with stathmin siRNA. Donwregulation of E-cadherin and up-regulation of vimetin in siRNA transfected cells is also confirmed.

marker, E-cadherin (Fig 25 Panels A & B). Downregulation of E-cadherin was also accompanied with a downregulation of p120 suggesting that E-Cadherin, vimentin and p120 levels are modulated by stathmin (Fig 25 Panels A).

Stathmin regulates TGFβ-mediated EMT through a Smad-independent mechanism in DU145 cells

Smad molecules are effectors of TGFβ signaling in the cell. As discussed in Chapter V, binding of the ligand to TGFβ Receptor Type II leads to the recruitment of TGFβ Receptor Type I and results in active receptor ligand complex. Subsequently, Smad2 and Smad3 is phosphorylated, to complex with Smad4, for subsequent translocation into the nucleus to activate transcription of target genes such as slug and snail. TGFβ can elicit the EMT phenotype through either a smad -dependent or – independent mechanim. The smad-dependent mechanism leads to the activation of genes such as snail and slug, which represses E-cadherin expression. The smad-independent mechanism can involve a number of molecules such as p38MAPK, RhoA, p160ROCK etc., and also suppress E-cadherin expression. We sought to identify which of these pathways is being modulated by stathmin in EMT. DU145 cells transfected either with control siRNA or with stathmin siRNA were treated with increasing concentrations of human recombinant TGF-β1 (0, 0.1, 0.5, 2.0, 5.0ng/ml) (Fig. 26 Panel A). Smad2 and Smad3 phosphorylation was studied by western blot analysis with antibodies raised against specific phospho-forms of Smad molecule (Fig. 26 Panel A). Interestingly, phosphorylated levels of Smad2 and Smad3 are similar at all concentrations tested irrespective of the presence or absence of stathmin expression (Fig. 26 Panel A). Since phospho-Smad2/3 levels were highest at 5.0ng/ml of TGF-β1, DU145 cells were treated

with this concentration for 0, 1, 2, 4 and 8 hours to determine the effects of stathmin expression on Smad2 and Smad3 phosphorylation over time (Fig. 26 Panel B). Irrespective of stathmin expression levels, TGF-β1 treatment caused Smad2 and Smad3 phosphorylation to increase with time up to 2 hours, after which it gradually decreased till no phosphorylation could be detected at 8 hours (Fig. 26 Panel B). These results indicate that stathmin regulates the TGF-β1-mediated EMT formation through a smad-independent mechanism.

Interestingly, although stathmin does not affect Smad2 and Smad3 phosphorylation, it does affect Smad2 sub-cellular localization in response to TGFβ treatment. To study Smad localization, DU145 cells transfected with control or stathmin siRNA were treated with TGFβ for 0, 1, 2 and 8 hours (Fig. 27). Smad2 localization was analyzed by immunocytochemistry. In cells transfected with control siRNA, TGFβ treatment caused nuclear translocation within one hour (Fig. 27f, g and h). However, in cells transfected with stathmin siRNA, Smad2 was localized in the cytoplasm even after 8 hours of TGFβ treatment (Fig. 27s). Hence, stathmin can modulate Smad2 nucleo-cytoplasmic shuttling in response to TGFβ treatment. To determine whether stathmin inhibits nuclear import or export of Smad2, cells were treated with 20nM Leptomycin B, which inhibits nuclear export of proteins with a nuclear export signal (NES). In DU145 cells transfected with stathmin siRNA, leptomycin B caused nuclear accumulation of Smad2 within one hour of TGFβ treatment (Fig. 27j). This mimics cellular localization of Smad2 in cells transfected with control siRNA and treated with TGFβ for one hour (Fig.

27f, g and h). Thus, stathmin does not affect nuclear import of Smad2. Rather, it modulates Smad2 localization by inhibiting/delaying its export from the nucleus.

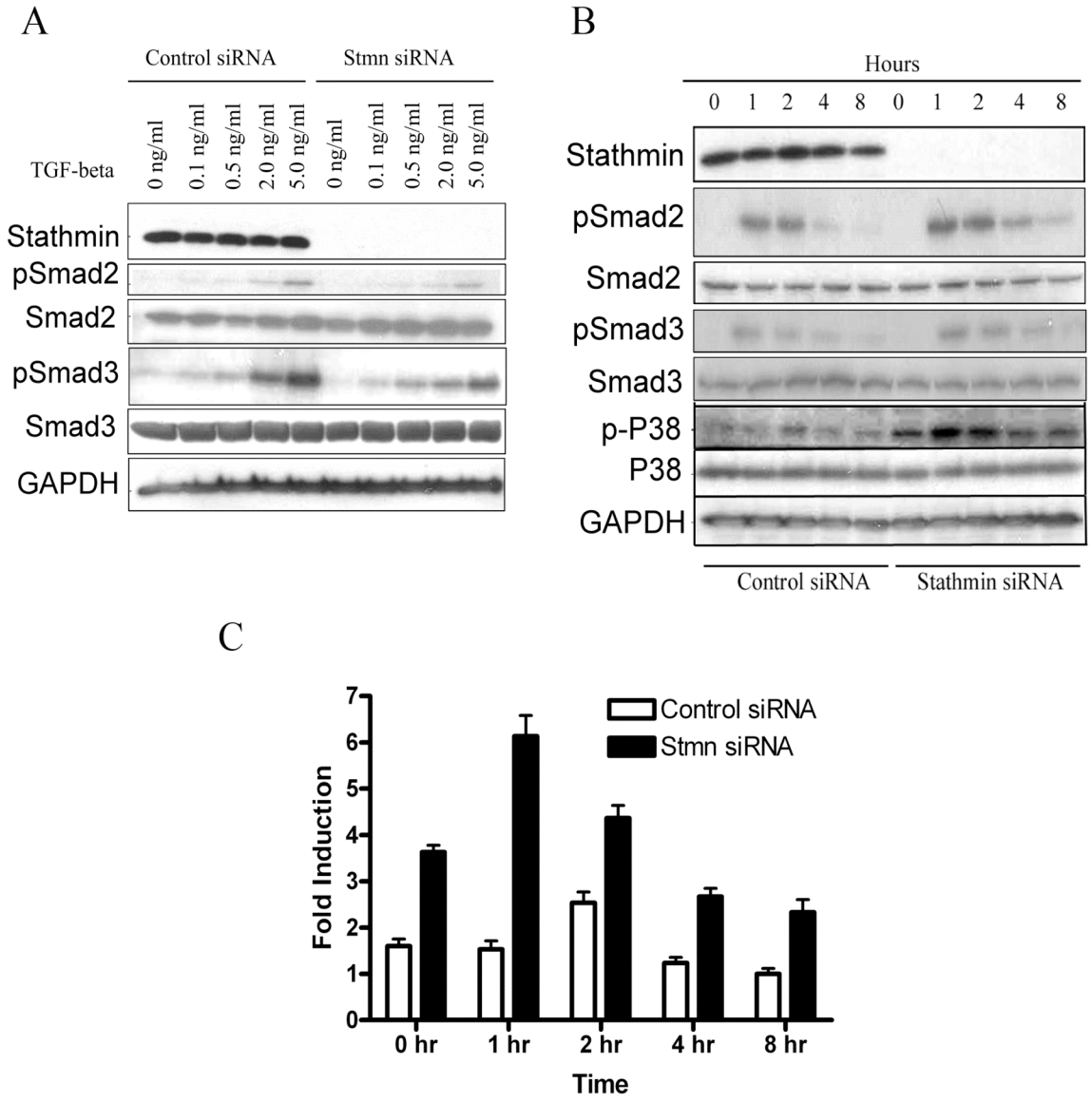

Figure 26. TGFβ-induced Smad2/3 and P38 phosphorylation in DU145 cells transfected with control or stathmin siRNA. A. Smad2 and Smad3 phosphorylation increases with increasing concentration of TGFβ (0-5ng/ml). Phosphorylation levels are similar in control- or stathmin-siRNA transfected cells. B. Smad2 and Smad3 phosphorylation is maximal after 1 hr of treatment with 5ng/ml TGFβ both in control- and stathmin-siRNA transfected cells. Phosphorylation at other time points are also similar between the two groups implying stathmin does not affect Smad phosphorylation. P38 phosphorylation at all time points is higher in stathmin-siRNA transfected cells, implying that stathmin downregulation can activate p38. C. Densitometric analysis reveals that stathmin downregulation results in a 4-fold induction in P38 phosphorylation in stathmin-siRNA cells compared to control-siRNA cells after 1 hr of TGFβ treatment.

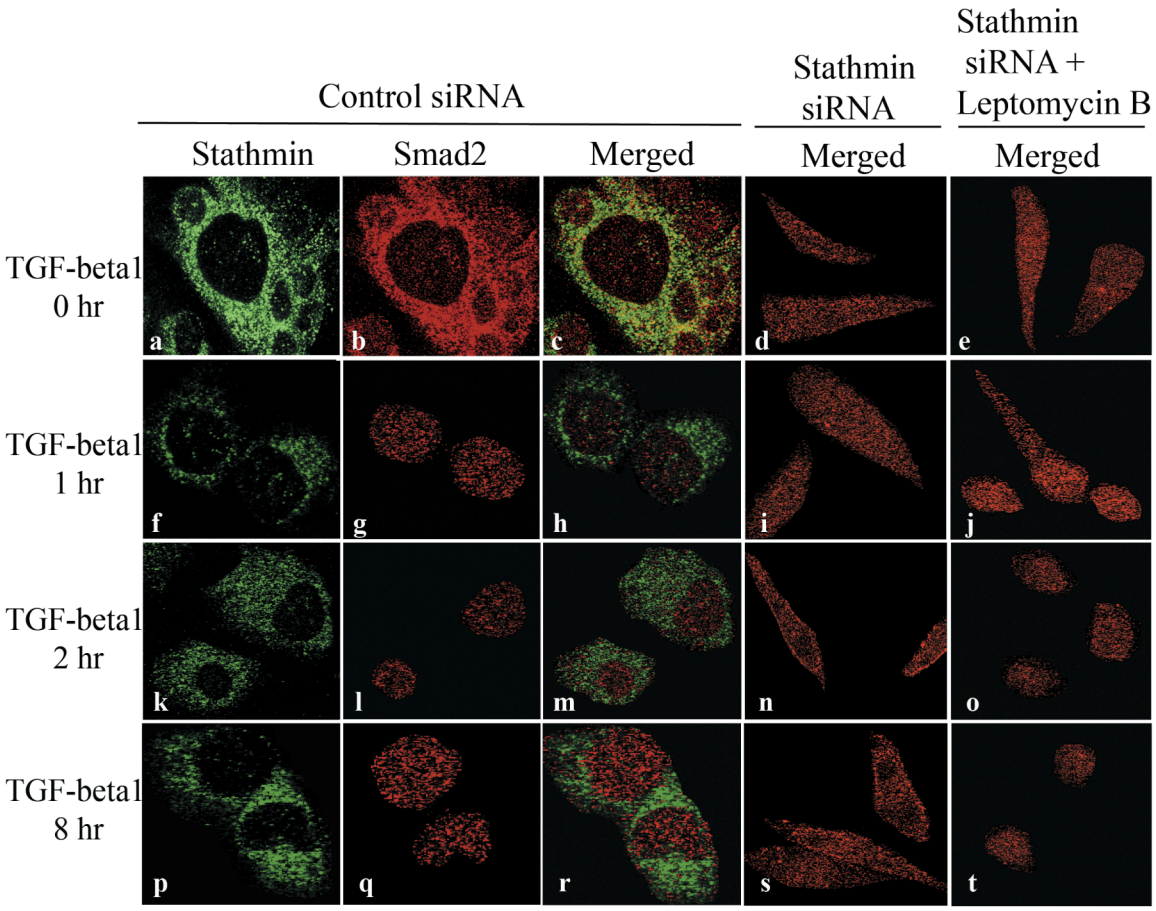

Figure 27. Stathmin modulates Smad2 cellular localization in response to TGFβ treatment. Control and stathmin-siRNA transfected DU145 cells were treated with 5ng/ml TGFβ (a-d, f-i, k-n, and p-s). Stathmin siRNA-transfected cells were treated with 20nM LeptomycinB (e, j, o and t). In control-siRNA transfected cells, Smad2 translocates to the nucleus within 1hr (f-h) of TGFβ treatment. However, in stathmin-siRNA transfected cells, Smad2 remains in the cytoplasm even after 8 hrs of TGFβ treatment (d, i, n and s). Twenty nanoMolar leptomycin B causes nuclear localization of Smad2 in response to TGFβ treatment in stathmin-siRNA transfected cells (e, j, o and t).

Stathmin modulates p38MAPK activity to regulate TGFβ-mediated EMT formation in DU145 cells

We next sought to determine if stathmin could modulate p38MAPK activity to elicit the EMT phenotype. TGFβ treatment has been reported to increase p38 phosphorylation in a time-dependent manner. As described earlier, DU145 cells transfected with control/stathmin siRNA were treated with 5.0ng/ml TGF-β1 for 0, 1, 2, 4 and 8 hours (Fig. 26 Panel B) and p38 phosphorylation was studied by western blot

analysis with an antibody that recognizes phosho-p38. p38 appears to be constitutively phosphorylated in stathmin siRNA-transfected DU145 cells, even in the absence of TGF-β1 treatment (Fig. 26 Panel B). Furthermore, p38 is maximally phosphorylated after 1hour (~4-fold compared to time 0 hour) (Fig. 26 Panel C) of TGF-β1 treatment and phospho-p38 decreases to basal levels by 4 hour. In contrast, phospho-p38 levels increased minimally at 2 hours post treatment in cells transfected with control siRNA. In cells transfected with stathmin siRNA, p38 phosphorylation was higher at all time-points tested compared to cells transfected with control siRNA (Fig. 26 Panel B and C).

These observations suggest that stathmin modulates p38 activity during EMT. If stathmin regulates EMT formation by modulating p38 activity, then inhibiting p38 phosphorylation should inhibit/delay appearance of EMT in DU145 cells transfected with stathmin siRNA. To test this hypothesis, we used the small molecule kinase inhibitor SB203580 to block p38 phosphorylation in DU145 cells transfected with stathmin or control siRNA (Fig. 28 Panels A and B). SB203580 has been reported to specifically block p38 phosphorylation. As described earlier, cells transfected with stathmin siRNA undergo EMT within 24 hours. In contrast, DU145 cells transfected with stathmin siRNA, but treated with SB203580 failed to transition into spindle-shaped cells indicative of the EMT phenotype (Fig. 28 Panel A). Western blot analysis confirms that p38 phosphorylation was inhibited by SB203580 (Fig. 28 Panel B). Double immunofluorescence was performed to determine the effects of inhibiting p38 phosphorylation on cell phenotype (Fig. 29). In stathmin siRNA-transfected DU145 cells treated with SB203580, E-cadherin is re-expressed and vimentin expression is lost

resulting in the restoration of the epithelial phenotype (Fig. 29). These data substantiate the observation that stathmin regulates TGF-β-mediated EMT by modulating p38MAPK activity.

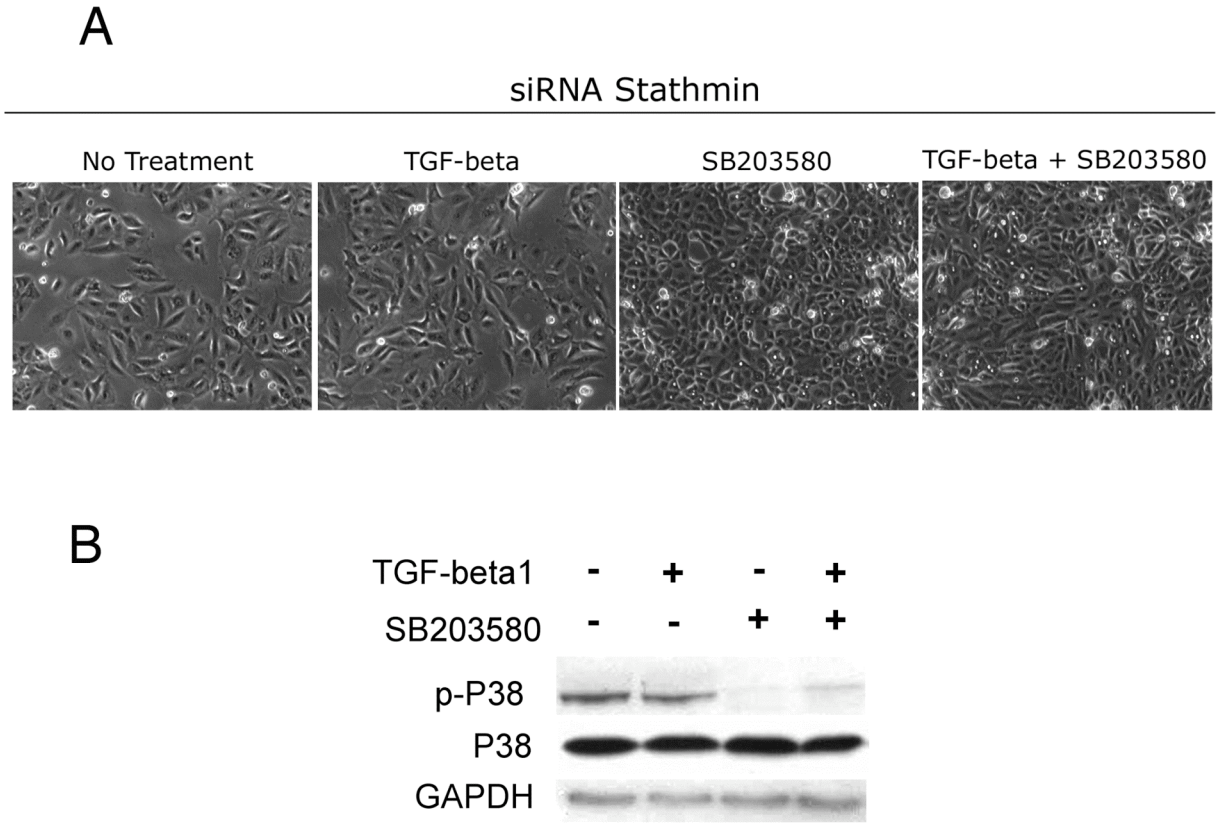

Figure 28. Blocking P38 activity inhibits EMT phenotype in stathmin-siRNA transfected DU145 cells. A. DU145 cells transfected with stathmin siRNA were treated with TGFβ and SB203580, either alone or in combination. Untreated cells and cells treated with TGFβ exhibit EMT phenotype within 24 hours of siRNA transfection. However cells treated with SB203580 alone or in combination with TGFβ do not undergo EMT. B. Western blot analysis to confirm that treatment with SB203580 results in downregulation of p38 phosphorylation. p38 levels do not change with treatment and GAPDH has been used to ensure equal loading in all the lanes.

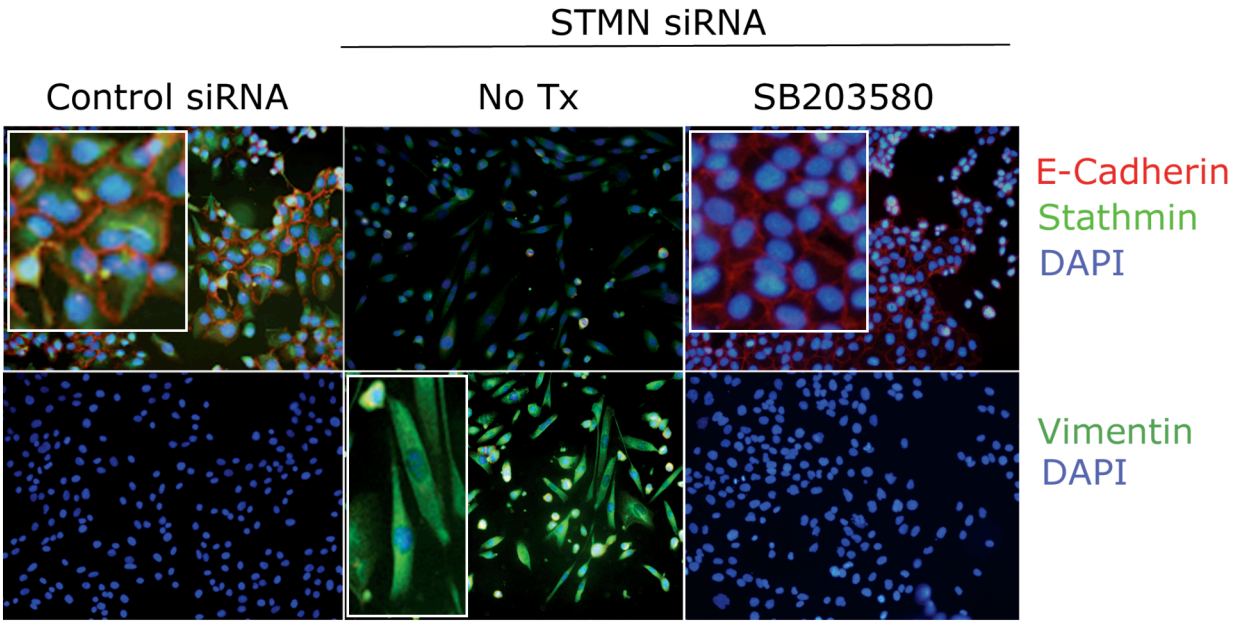

Figure 29. Inhibiting p38 phosphorylation restores epithelial phenotype in DU145 cells transfected with stathmin-siRNA. Cells transfected with control siRNA express E-cadherin and stathmin but do not express Vimentin. Cells transfected with stathmin siRNA express Vimentin but do not express E-cadherin and stathmin. However, inhibiting P38 activation by using SB203580 results in the reexpression of E-cadherin and downregulation of Vimentin, implying epithelial phenotype is restored in absence of stathmin.

Stathmin modulates TGFβ-mediated EMT formation in murine normal mammary NMuMG cells

The mouse mammary epithelial cell line NMuMG has been extensively utilized to study the EMT phenotype. These cells respond to TGF-β1 treatment within 24 hours with a change in cell morphology, delocalization of E-cadherin and zonula occludens-1 from cell-cell junctions and formation of actin stress fibers typical of EMT (170). Furthermore, TGF-β1 treatment phosphorylates p38 and induces EMT in NMuMG cells (176). Zhang et al. have used a mutant TGFβ Receptor I which is deficient in its ability to bind Smad proteins, but retains its kinase activity, to demonstrate that TGFβ activates p38 through a Smad-independent mechanism in eliciting the EMT phenotype (176). Interestingly,

western blot analysis reveals that NMuMG cells either lack or has very low levels of stathmin expression (Fig. 30 Panel B). If stathmin regulated modulation of p38 activity is a general mechanism in inducing EMT phenotype, overexpression of stathmin should delay or inhibit TGFβ-mediated EMT in NMuMG cells. We used a Flag-tagged full-length mouse stathmin cDNA construct in pDream2.1 vector to transiently overexpress the protein in NMuMG cells. Cells transfected with empty vector was used as controls. NMuMG cells transfected with stathmin cDNA or empty vector were treated with 5.0ng/ml of TGFβ for 24 hours. Cells transfected with vector carrying stathmin cDNA failed to elicit EMT phenotype and maintained an epithelial phenotype after 48 hours of TGFβ treatment (Fig. 30 Panel A). In contrast, EMT was observed in control cells by 18 hours (Fig. 30 Panel A). Western blot analysis confirms that the overexpression of stathmin in the transfected cells inhibits TGF-β1 induced p38 phosphorylation (Fig. 30 Panel B).

Hence, our results indicate that stathmin inhibits p38 phosphorylation and conserves the epithelial phenotype, whereas loss of stathmin up-regulates p38 phosphorylation and induces EMT. Furthermore, these results demonstrate that stathmin can modulate TGFβ-mediated EMT formation both in tumor (DU145) and normal (NMuMG) cells. This, to our knowledge, is the first study to establish the regulation of stathmin expression as a general mechanism in eliciting EMT phenotype in prostate and breast cell lines.

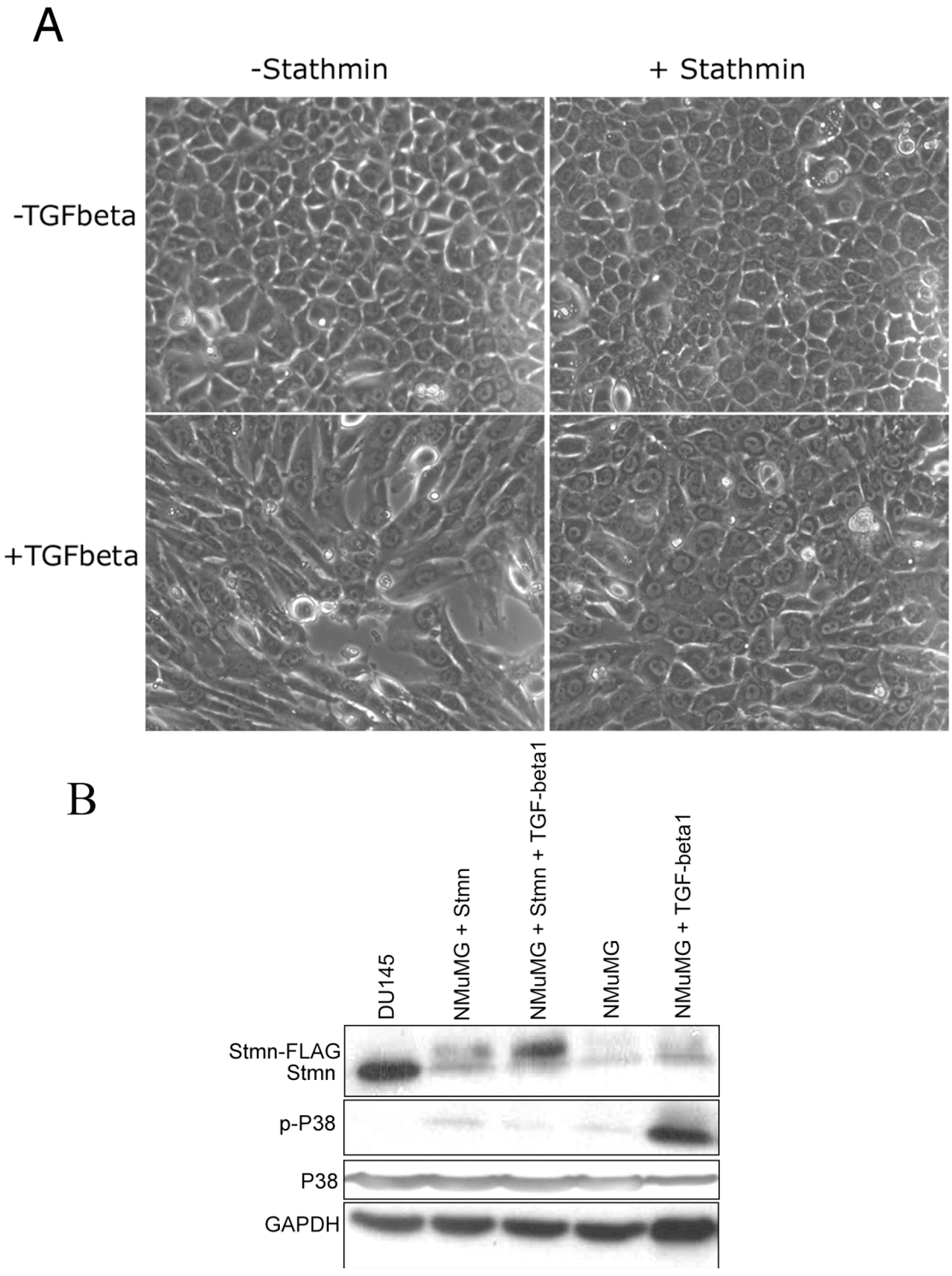

Figure 30. Overexpression of stathmin in NMuMG cells prevents TGFβ-mediated EMT. A. Full-length Flag-tagged mouse stathmin cDNA or empty vector is overexpressed in NMuMG cells. Cells with empty vector undergo EMT in response to TGFβ treatment within 18 hours. Cells overexpressing stathmin do not undergo EMT even after 48 hours of TGFβ treatment. B. Western blot analysis exhibits increased stathmin expression and impaired p38 phosphorylation in transfected cells. In cells transfected with empty vector 5ng/ml TGFβ treatment induces p38 phosphorylation.