

TGF- β 1 regulates cell fate during epithelial–mesenchymal transition by upregulating survivin

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Members of the transforming growth factor beta (TGF- β) superfamily are multifunctional cytokines that regulate several cellular processes, including cell cycle arrest, differentiation, morphogenesis, and apoptosis. TGF- β promotes extracellular matrix production and morphological change. Morphogenetic responses to TGF- β include cell migration and epithelial–mesenchymal transition (EMT), which are critical during embryogenesis, development of fibrotic diseases, and the spreading of advanced carcinomas. The purpose of this study was to clarify how TGF- β regulates the fate of retinal pigment epithelial (RPE) cells. TGF- β 1 promoted cell cycle progression and phosphorylation of retinoblastoma protein (Rb) in ARPE-19 cells. TGF- β 1 induced survivin expression, which in turn stabilized tubulin and Aurora B. RT-PCR and western blot analysis revealed that survivin expression increased in ARPE-19 cells following TGF- β 1 treatment. When survivin was depleted, TGF- β 1 induced cell cycle arrest and apoptosis and also reduced Rb phosphorylation. In conclusion, the present study shows that induction of EMT in human RPE cells upregulates survivin, leading to survivin-dependent inhibition of cell cycle arrest and apoptosis. Whether cells undergo EMT or apoptosis in response to TGF- β 1 is dependent on their cell cycle state, and TGF- β 1 regulates the cell cycle via survivin.

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Transforming growth factor beta (TGF- β) is a major inducer of epithelial–mesenchymal transition (EMT) during development, fibrosis, and carcinogenesis. Different isoforms of TGF- β mediate various effects depending on the specific cellular context.¹ TGF- β promotes extracellular matrix production and induces changes in cell morphology. Morphogenetic responses to TGF- β isoforms include cell migration and EMT.^{2–4} EMT is characterized by the disassembly of cell–cell contacts, remodeling of the actin cytoskeleton, and separation of cells, which generates fibroblast-like cells that express mesenchymal markers and migratory properties.^{5–8} TGF- β 1 was first described as an inducer of EMT in normal mammary epithelial cells,⁹ and has since been shown to mediate EMT in various epithelial cells *in vitro*, including renal proximal tubular, retinal, lens, and alveolar epithelial cells.^{10–13}

Furthermore, TGF- β induces apoptosis in several cell types including hepatocytes and hepatomas.¹⁴ On the other hand, TGF- β has an anti-apoptotic function and can promote cell survival, proliferation, and differentiation.¹⁵ The ability of cells to evade TGF- β -induced growth arrest and/or apoptosis results in uncontrolled, autonomous cell growth.^{16,17} Studying the signaling mechanism(s) through which the diverse effects of TGF- β are mediated is therefore crucial to better understand various cellular processes, and may provide the basis for novel disease treatments.

TGF- β and its signaling pathways, which comprise a complex signaling network, have been the focus of numerous studies.¹⁸ The effects of TGF- β vary according to the cell type and the environmental and physiological conditions. Inhibition of TGF- β signaling in T cells leads to spontaneous T-cell differentiation and autoimmune disease,^{19,20} indicating that TGF- β signaling is required for T-cell homeostasis. TGF- β signaling is disrupted in some tumors and cancer cells, and TGF- β strongly inhibits the proliferation of epithelial cells.²¹

The receptors that mediate TGF- β signaling are well studied. Signaling downstream of TGF- β receptor binding is mediated by Smads, and their interactions have been intensively studied and characterized over the past several years. The ERK, JNK, and p38 MAP kinases regulate TGF- β -mediated signaling. Crosstalk between different components of the TGF- β signaling pathway may explain the diverse range of effects mediated by TGF- β .

Several pathways, involving many downstream proteins, mediate the effects of TGF- β 1. Many critical steps in intracellular TGF- β signaling are mediated by Smad proteins. However, Smad-independent signaling transduction pathways are also involved in the biological activities of TGF- β .²² As the Smad pathway principally regulates gene expression, it was originally thought that non-Smad effectors mediate the rapid or direct effects of TGF- β on the actin cytoskeleton. However, we previously suggested that the Smad pathway

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Abbreviations: EMT, epithelial–mesenchymal transition; PI, propidium iodide; Rb, retinoblastoma protein; RPE, retinal pigment epithelial; siRNA, small interfering RNA; TGF- β 1, transforming growth factor β 1

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has a critical role in TGF- β 1-induced actin rearrangement by regulating the RhoA GTPase. Smad3 regulates RhoA activation via NET1 by controlling the mRNA and protein expression of RhoA and the cytoplasmic localization of NET1.²³

Apoptosis is a mechanism of programmed cell death that maintains cell homeostasis in multicellular organisms. This process is regulated via an exquisite balance between proapoptotic proteins and anti-apoptotic proteins. Survivin was initially described as a member of the inhibitor of apoptosis (IAP) family, which contains a single baculoviral IAP repeat domain²⁴ and is now known to be one of the most tumor-specific genes in the human genome.²⁵ Survivin forms a complicated interaction network and regulates several cell processes,²⁶ such as apoptosis, the spindle checkpoint system,^{27,28} microtubule dynamics,²⁹ and the cellular stress response.³⁰ Furthermore, survivin is a component of the chromosome passenger complex^{31,32} and has a critical role in the regulation of mitosis.³³ The mechanism by which survivin inhibits apoptosis remains unclear.

Rapid progress is being made to characterize the diverse effects of TGF- β and the underlying mechanisms by which these effects are mediated; however, relatively little is known about the signaling mechanism(s) responsible for the apoptotic, anti-apoptotic, and proliferative effects mediated by TGF- β . In this report, we studied the effects of TGF- β 1 on apoptosis and EMT in ARPE-19 cells. EMT induction of ARPE-19 cells by TGF- β correlated with an anti-apoptotic effect that regulated cell cycle progression. This indicated that cells either underwent EMT or apoptosis in response to TGF- β 1 treatment. We next investigated why cells respond differently to TGF- β 1 under the same experimental conditions. This is likely due to the differences that lie in themselves. Indeed, the cell cycle regulates whether cells undergo apoptosis or EMT in response to TGF- β 1.³⁴

Here, we investigated the role of survivin in determining whether a cell survives or undergoes apoptosis in response to TGF- β 1 by depleting survivin levels using small interfering RNA (siRNA). We propose that survivin has a critical role in TGF- β 1-induced EMT by regulating the cell cycle and tubulin stability. We also demonstrate that TGF- β determines cell fate by modulating survivin expression. These results provide evidence for a novel mechanism underlying the regulation of cell fate by TGF- β 1, which is dependent on the modulation of the cell cycle and tubulin stability by survivin.

Results

Retinal pigment epithelial cells survive during TGF- β 1-induced EMT. TGF- β 1 treatment for 48 h led to dramatic morphological changes and stimulated N-cadherin and fibronectin protein content in the spontaneously immortalized human retinal pigment epithelial (RPE) cell line, ARPE-19 (Figures 1a and b). TGF- β -treated ARPE-19 cells were larger and less compact than untreated cells. To determine whether TGF- β 1 induced cell death in human RPE cells, we examined the viability of ARPE-19 cells cultured for 48 h in DMEM containing TGF- β 1 in a CCK-8 assay. The number of viable cells increased significantly following incubation with TGF- β 1 for 24 h (Figure 1c).

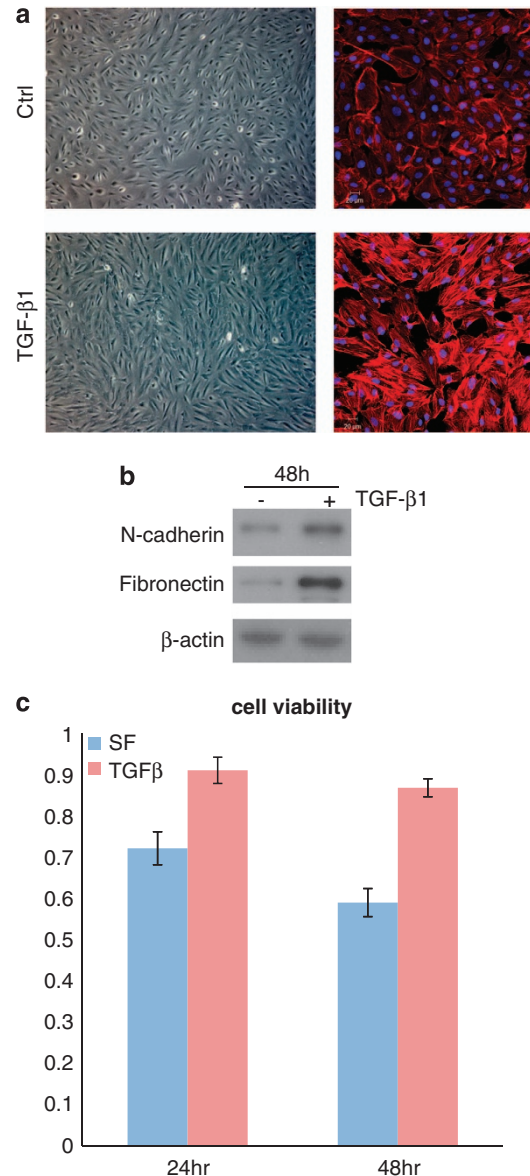


Figure 1 TGF- β 1 induces cell cycle progression and EMT. Serum-starved ARPE-19 cells were cultured in the absence or presence of 10 ng/ml TGF- β 1 for 24 or 48 h. (a) EMT was examined by morphological changes and the actin cytoskeleton was stained with Rhodamine-labeled phalloidin. *Ctrl*, control. Bar, 20 μ M. (b) Cell lysates were prepared and the level of protein was analyzed by western blotting with a specific antibody. (c) Cell viability was measured 24 and 48 h after TGF- β 1 treatment using the CCK-8 assay

Cell cycle progression is unaffected and apoptosis is inhibited in RPE cells during TGF- β 1-induced EMT. As TGF- β 1-treated cells survived during EMT, we next investigated the role of TGF- β 1 in the cell cycle. To examine whether TGF- β 1 affects cell cycle progression in human RPE cells, the proportion of cells in different phases of the cell cycle was determined by flow cytometry. TGF- β 1 treatment did not arrest the cell cycle in ARPE-19 cells. This indicates that TGF- β 1 leads to undergo cell cycle progression (Figure 2a). Flow cytometric analysis of ARPE-19 cells treated for 24 h with TGF- β 1, followed by incubation with

Annexin V-FITC and propidium iodide (PI), showed the apoptotic fraction by TGF- β 1. The percentage of apoptotic cells was determined by dual parameter analysis (Figure 2b). TGF- β 1 did not increase the number of apoptotic cells compared with control cells (Figure 2b). In summary, TGF- β 1 did not disrupt cell cycle progression or induce apoptosis in ARPE-19 cells.

Cyclin D is the first cyclin produced during the cell cycle in response to extracellular signals. Cyclin D binds to CDK4, forming the active cyclin D-CDK4 complex. The cyclin D-CDK4 complex phosphorylates and inactivates the retinoblastoma susceptibility protein (Rb). Hyperphosphorylated Rb dissociates from the E2F/DP1/Rb complex, leading to E2F activation. The activation of E2F results in the transcription of various genes, including cyclin E, cyclin A, DNA polymerase, and thymidine kinase. Cyclin E binds CDK2, forming the cyclin E-CDK2 complex, which then promotes progression from G₁ to S phase. To further examine cyclin-CDK kinase activity and to determine whether the TGF- β 1-induced proliferation of ARPE-19 cells is mediated by Rb, western analysis was performed using an antibody that specifically recognizes phosphorylated Rb. Treatment of ARPE-19 cells with TGF- β 1 increased the level of hyperphosphorylated Rb, which indicates that Rb was inactivated following TGF- β 1 treatment (Figure 2c). Furthermore, the level of Rb phosphorylated at serine 780 was increased following TGF- β 1 treatment. This site is critical for the activation of Rb and this result confirms that TGF- β 1 inhibits Rb. The level of cyclin D1 increased significantly in a time-dependent manner following TGF- β 1 treatment (Figure 2c). Rb is at least partly phosphorylated by cdk2. For cdk2 to be activated, it must bind a cyclin. TGF- β 1 increased the active form of cdk2 (Figure 2c). Phosphorylation at threonine 160 induces a shift in the electrophoretic mobility of cdk2.³⁵ The tyrosine 15 and threonine 14 residues of cdk2 are dephosphorylated by the phosphatase cdc25A.³⁶ Cdc25 phosphatases promote cell cycle progression by dephosphorylating and activating cdk2, which are the major driving force for cell cycle progression.^{37,38} Cdc25A activates cyclin E (A)-Cdk2 during G₁ and S phase, and also appears to

be involved in the activation of Cdk1 at the G₂/M checkpoint.^{39,40} The level of cdc25A increased following TGF- β 1 treatment (Figure 2c). Cyclin E associates with and activates cdk2 in G₁ phase. The increase in the levels of Rb hyperphosphorylation and cyclin D1 was greater when cells were treated with TGF- β 1 for 6 h than when they were treated for 48 h (data not shown). The levels of the other proteins discussed above were similar, regardless of whether the cells were treated with TGF- β 1 for 24 or 48 h (data not shown). These data indicate that TGF- β 1 induces cell cycle progression by regulating the activity and expression of several cell cycle regulators.

TGF- β induces survivin expression. As survivin inhibits apoptosis, we hypothesized that the treatment with TGF- β 1 might upregulate survivin. To test this, we performed PCR and western blot analyses on ARPE-19 cells treated with TGF- β 1 for different lengths of time. The expression of survivin mRNA increased following TGF- β 1 treatment (Figure 3a). Survivin protein levels also increased in TGF- β 1-treated cells in a time-dependent manner (Figure 3a). This increase was observed after 30 min of TGF- β 1 treatment and peaked after 6 h.

Survivin regulates TGF- β 1-induced cell cycle progression. As TGF- β 1 was previously shown to upregulate survivin, we hypothesized that survivin may contribute to the cell cycle progression of ARPE-19 cells treated with TGF- β 1. To test this hypothesis, the functional effects of suppressing expression of the *survivin* gene in ARPE-19 cells were determined using siRNA. Four siRNA duplexes were designed to target each transcript, and gene silencing was confirmed using RT-PCR (data not shown). The duplex that most effectively reduced *survivin* expression was used in all subsequent experiments and that survivin siRNA markedly reduced survivin mRNA in ARPE-19 cells *in vitro* by ~75% compared with control siRNA treatment groups.

When survivin expression was reduced, the cells had significantly increased G₂/M phase in comparison with control

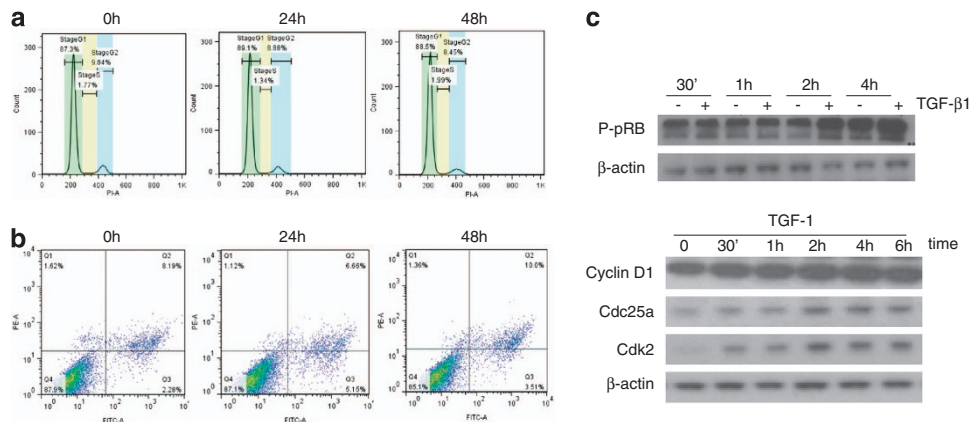


Figure 2 TGF- β 1 induces cell cycle progression. (a) Samples were taken after 24 and 48 h of TGF- β 1 treatment, and DNA content was assayed by flow cytometry and PI staining (FL2-A). The gates and percentages of cells in the sub-G₁ (<G₁), G₁ and G₂ phases are indicated. (b) TGF- β does not affect apoptosis in ARPE-19 cells. Samples were taken 24 and 48 h of TGF- β 1 treatment, and cells were stained with Annexin V and PI and analyzed by FACS. (c) Activation of cell cycle regulators by TGF- β 1. Serum-starved ARPE-19 cells were cultured in the absence or presence of 10 ng/ml TGF- β 1 for 0.5–6 h. Cell lysates were analyzed by western blotting with a specific antibody

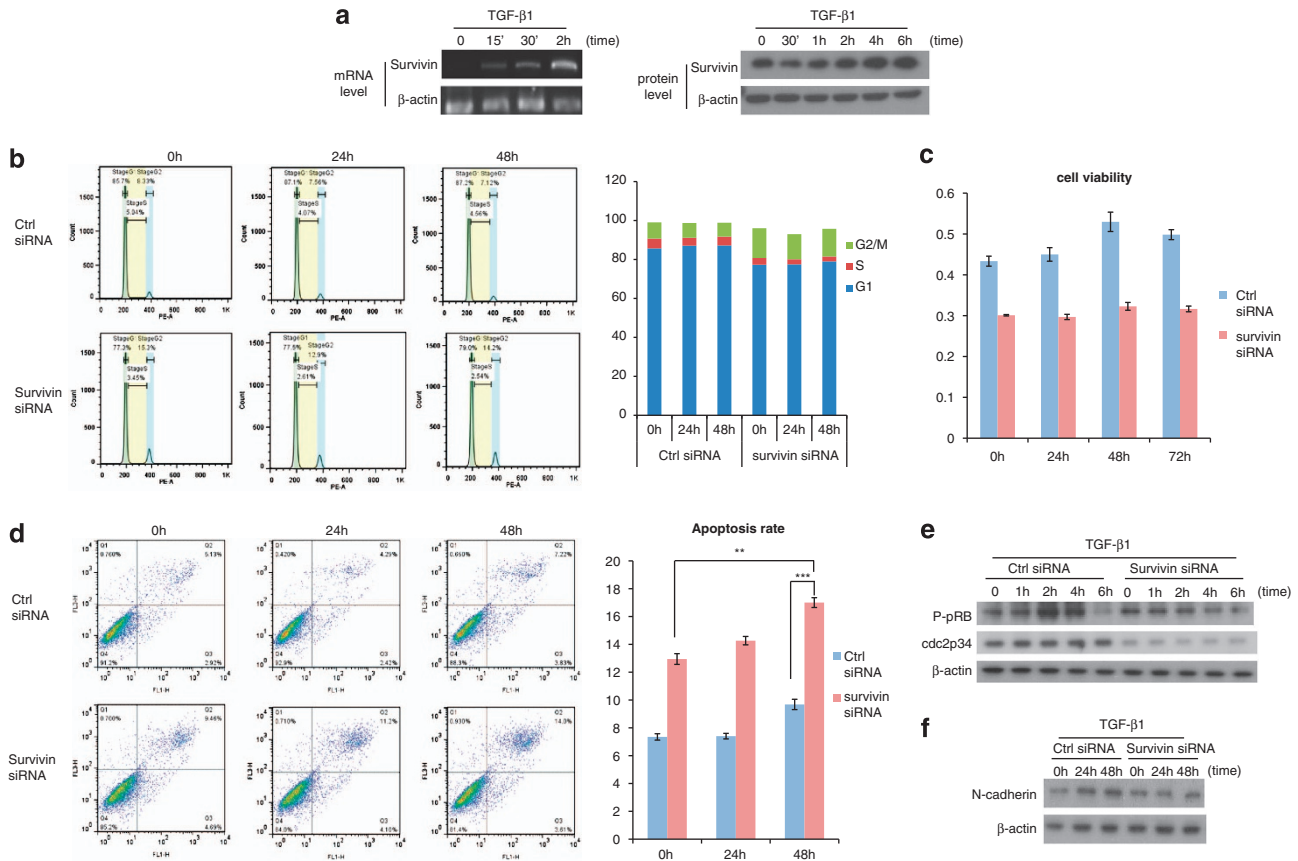


Figure 3 Survivin is critical for TGF- β 1-mediated cell cycle progression and EMT. **(a)** TGF- β 1 induces survivin expression in ARPE-19 cells. Serum-starved ARPE-19 cells were cultured in the absence or presence of 10 ng/ml TGF- β 1 for different times. Total RNA was isolated, reverse transcribed, and amplified by PCR. Cell lysates were prepared and the level of survivin protein was analyzed by western blotting with a specific antibody. **(b–f)** ARPE-19 cells were transfected with control or survivin-targeted siRNA. After 12 h, cells were switched to serum-free medium for 3 h and treated with 10 ng/ml TGF- β 1 for 24 or 48 h. **(b)** Survivin regulates TGF- β 1-mediated cell cycle progression. Samples were taken after 48 h of TGF- β 1 treatment and the DNA content was assayed by flow cytometry and PI staining (FL2-A). The gates and percentages of cells in the sub-G $_1$ (<G $_1$), G $_1$, and G $_2$ phases are indicated. **(c)** Depletion of survivin reduces the viability of TGF- β 1-treated cells. Cell viability was measured after treatment with TGF- β 1 for 24 and 48 h using the CCK-8 assay. **(d)** Depletion of survivin induces TGF- β 1-mediated apoptosis. Samples were taken after cells had been treated with TGF- β 1 for 24 h and the cells were stained with Annexin V and PI, and analyzed by FACS. *** P < 0.001 and ** P < 0.01 (Student's t -test). **(e)** Depletion of survivin reduces Rb phosphorylation. Cell lysates were analyzed by western blotting with a specific antibody. *Ctrl*, control. **(f)** Depletion of survivin reduces N-cadherin. Cell lysates were analyzed by western blotting with a specific antibody. *Ctrl*, control

cells (Figure 3b). Cell viability was reduced (Figure 3c) and TGF- β 1-induced apoptosis increased (Figure 3d) when survivin was depleted (** P = 0.0089). These data demonstrate that upregulation of survivin promotes cell cycle progression and that this is required for TGF- β 1-induced EMT.

Rb hyperphosphorylation is critical for cell to cell cycle progress. To further demonstrate the role of survivin in TGF- β 1-induced EMT in ARPE-19 cells, we studied the effect of survivin depletion on Rb phosphorylation. TGF- β 1 increased the levels of the hyperphosphorylated forms of Rb, and this effect was reduced when survivin was depleted (Figure 3e). The increase in cdc2 levels induced by TGF- β 1 was blocked when survivin was depleted (Figure 3e). Interestingly, the increase in N-cadherin levels induced by TGF- β 1 was partially prevented by blocking survivin.

Downregulation of survivin by TGF- β 1 induces cell apoptosis in Hep3B cells. To determine the effect of

survivin on TGF- β 1-induced cell fate decision, we selected Hep3B cells and then examined the level of survivin expression on Hep3B cells treated with TGF- β 1 for different lengths of time. The expression of survivin protein decreased in TGF- β 1-treated cells in a time-dependent manner (Figure 4a). Furthermore, the number of apoptotic cells increased in TGF- β 1-treated Hep3B cells compared with control cells (Figure 4b). TGF- β 1 treatment induced cell cycle arrest in ARPE-19 cells. TGF- β 1-treated Hep3B cells had significantly increased G $_2$ /M phase in comparison with control cells (Figure 4c). These data demonstrate that downregulation of survivin promotes cell cycle arrest and that this is required for TGF- β 1-induced apoptosis. In conclusion, cells downregulating survivin by TGF- β 1 induce not EMT but apoptosis.

TGF- β 1-induced apoptosis and EMT are linked with the cell cycle. We investigated whether apoptosis and EMT in response to TGF- β 1 are influenced by cell cycle status.

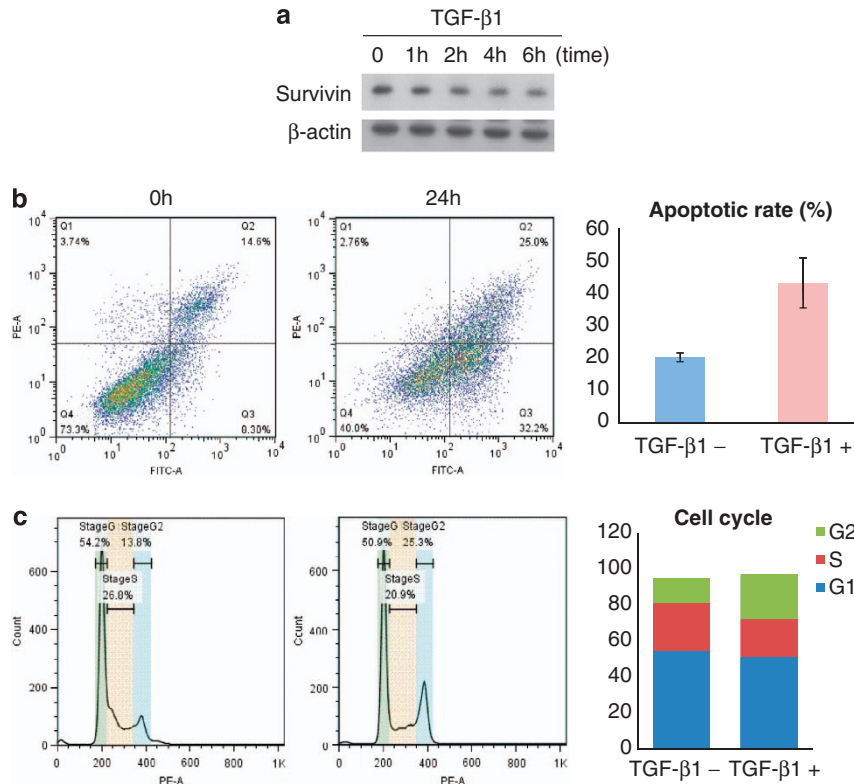


Figure 4 TGF- β 1 induces apoptosis and downregulates survivin in Hep3B cells. (a) TGF- β 1 downregulates survivin. Serum-starved Hep3B cells were cultured in the absence or presence of 10 ng/ml TGF- β 1 for 1–6 h. Cell lysates were prepared and the level of survivin protein was analyzed by western blotting with a specific antibody. (b) TGF- β 1 induces apoptosis and in Hep3B cells. Samples were taken 24 h of TGF- β 1 treatment, and cells were stained with Annexin V and analyzed by FACS. (c) TGF- β 1 induces cell cycle arrest in Hep3B cells. Samples were taken 24 h of TGF- β 1 treatment, and cells were stained with PI and analyzed by FACS

We synchronized cells in G1/S or G2/M phase and examined EMT and apoptosis in response to TGF- β 1. TGF- β 1 induced apoptosis in cells synchronized in G2/M phase (Figure 5b). These data demonstrate that cells arrested in G2/M phase undergo apoptosis in response to TGF- β 1.

TGF- β 1 regulates cell mitosis and microtubule stability through survivin. In addition to regulating apoptosis, and similar to the other members of the IAP family, survivin also regulates cell cycle progression during mitosis. We hypothesized that the ability of TGF- β 1 to induce cell cycle progression was dependent upon survivin. To investigate the role of survivin in TGF- β 1-induced EMT, we investigated the effects of survivin on mitosis and the mitotic kinase, Aurora B. First, we evaluated the level of acetylated α -tubulin in cells, which is an indicator of microtubule stability. The level of acetylated α -tubulin increased following TGF- β 1 treatment, indicating that the microtubules were more stable; this effect was not seen in cells depleted of survivin (Figure 6a). Furthermore, we found that TGF- β 1-induced mitosis increased by upregulating survivin. In Figure 6a, we can see multiple mitotic processes, including prophase, metaphase, and telophase with survivin in TGF- β 1-treated cells. In this figure, we have shown that survivin regulated kinetochore–microtubule interactions. From these results, we found that TGF- β 1 treatment increase mitosis, and survivin should act as a key molecule in TGF- β 1-induced mitosis.

Survivin can interact with Aurora B directly.⁴¹ TGF- β 1 treatment induced Aurora B, an effect that was not seen following the depletion of survivin (Figure 6b). These results indicate that survivin, which is upregulated in response to TGF- β 1, not only directly binds but also stabilizes Aurora B.

Role of PI3 kinase in the upregulation of survivin in response to TGF- β 1. To determine the key signaling mediator responsible for the upregulation of survivin in response to TGF- β 1, we used kinase inhibitors to individually block each signaling pathway in ARPE-19 cells treated with TGF- β 1, and then examined the level of survivin expression. Inhibition of MEK or PI3K blocked the upregulation of survivin following TGF- β 1 treatment, whereas the inhibition of Rho did not (Figure 7). These data suggest that PI3 kinase signaling is important for the upregulation of survivin in response to TGF- β 1 in APRE-19 cells.

Discussion

TGF- β is a multifunctional growth factor that regulates cell fate, including EMT and apoptosis. We previously reported that TGF- β 1 induces cytoskeletal actin rearrangement in human RPE cells via Rho GTPase-dependent pathways that modulate the activities of LIM kinase and cofilin.¹³ We also showed that TGF- β 1 strongly induces the Smad3 pathway, and that RhoA is not required downstream for

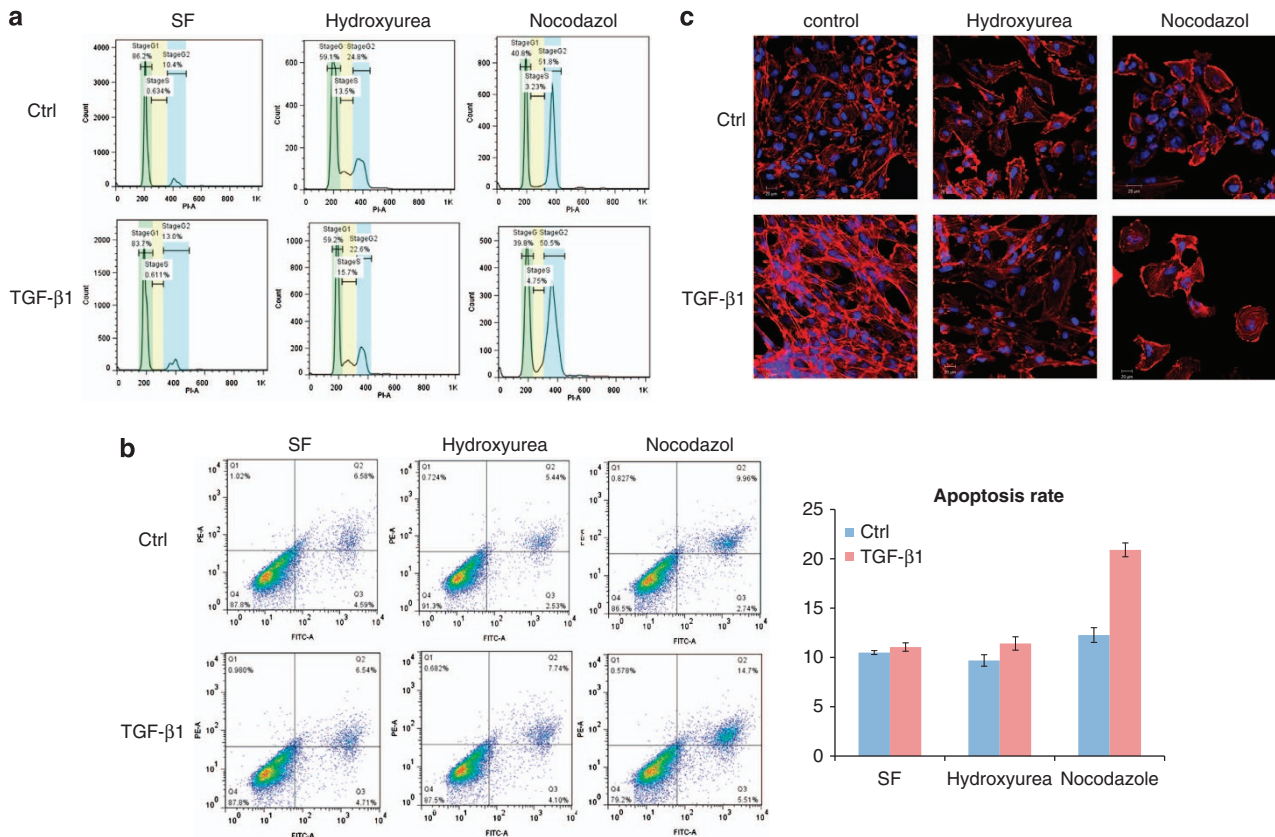


Figure 5 TGF- β 1-induced EMT and apoptosis are dependent on cell cycle status. Non-synchronized cells and cells synchronized at G1/S or G2/M were treated with 10 ng/ml TGF- β 1 for 36 h. (a) Samples were taken 48 h later and the DNA content was assayed by flow cytometry and PI staining (FL2-A). The gates and percentages of cells in the sub-G₁ (< G₁), G₁, and G₂ phases are indicated. (b) Samples were taken 24 h later and the cells were stained with Annexin V and PI and analyzed by FACS. SF, Serum free. Ctrl, control. (c) EMT was examined by morphological changes and the actin cytoskeleton was stained with Rhodamine-labeled phalloidin. Ctrl, control. Bar, 20 μ M

TGF- β 1-induced Smad3 activation but acts as downstream of Smad3 via NET^{1,23}. In the present study, we report that TGF- β 1 signaling upregulates survivin to inhibit apoptosis during EMT. TGF- β 1 led to both EMT and cell cycle progression, but not apoptosis, in ARPE-19 cells (Figures 1 and 2). Treatment of ARPE-19 cells with TGF- β 1 increased the level of hyperphosphorylated Rb, which indicates that Rb was inactivated following TGF- β 1 treatment (Figure 2c). Moreover, the level of Rb phosphorylated at serine 780 and the level of cyclin D1 were increased following TGF- β 1 treatment (Figure 2c). Cyclin D is the first cyclin produced during the cell cycle in response to extracellular signals. Cyclin D binds to CDK4, forming the active cyclin D-CDK4 complex, the cyclin D-CDK4 complex phosphorylates and inactivates the Rb. Hyperphosphorylated Rb dissociates from the E2F/DP1/Rb complex, leading to E2F activation. The activation of E2F results in the transcription of various genes, including cyclin E, cyclin A, DNA polymerase, and thymidine kinase. Rb is at least partly phosphorylated by cdk2. For cdk2 to be activated, it must bind a cyclin. Cyclin E binds CDK2, forming the cyclin E-CDK2 complex, which then promotes progression from G₁ to S phase. In this study, we showed that TGF- β 1 increased the active form of cdk2 and the level of cdc25A (Figure 2c). Cdc25 phosphatases promote cell cycle progression by dephosphorylating and activating cdk. As a

result, we prove that TGF- β 1 induces cell cycle progression by regulating the activity and expression of several cell cycle regulators in this study.

As it is well known that cell cycle progression is associated with alterations in cellular components and corresponding signaling events, there might be a link between cell cycle progression and TGF- β 1-induced apoptosis and EMT. TGF- β 1 treatment led to the upregulation of survivin, an IAP, which correlated with enhanced cell survival (Figure 3). On the other hand, Hep3B cells downregulated survivin by TGF- β 1 increased G₂/M arrest and apoptosis (Figure 4). These results indicate that depending on whether the survivin upregulated or downregulated by TGF- β 1 determines cell fate for EMT or apoptosis.

Survivin is a member of the IAP family and is a key regulator of mitosis and programmed cell death. Depletion of survivin using siRNA significantly enhanced TGF- β 1-induced apoptosis and cell cycle arrest (Figure 3). We tested whether this effect was correlated with the cell cycle status of the cells. We synchronized cells in G₂/M phase and examined the level of apoptosis following TGF- β 1 treatment. Interestingly, TGF- β 1 induced apoptosis in cells synchronized in G₂/M phase (Figure 5). These results indicate that the cell cycle stage influences whether cells undergo EMT or apoptosis in response to TGF- β 1. This may explain why TGF- β 1 treatment

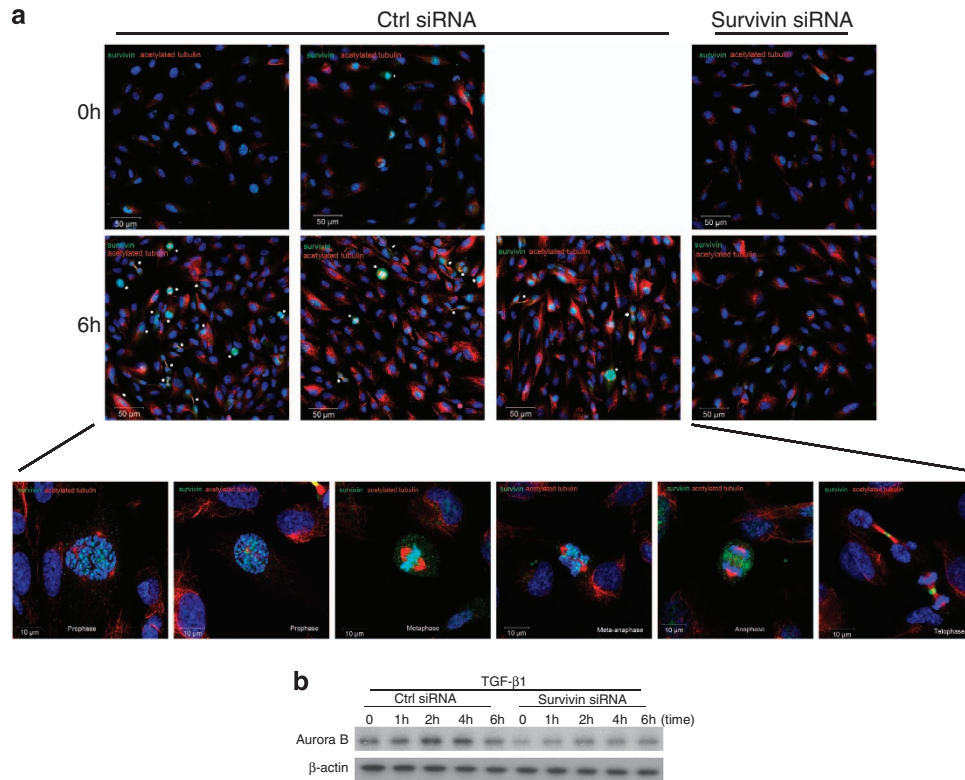


Figure 6 TGF- β 1 induces tubulin stability and Aurora B stabilization in ARPE-19 cells. ARPE-19 cells were transfected with control or survivin-targeted siRNA. (a) TGF- β 1 increases tubulin stability. ARPE-19 cells were cultured in the absence or presence of 10 ng/ml TGF- β 1 for 6 h and then fixed and stained with an anti-acetylated tubulin antibody (red) and anti-survivin antibody (green). Nuclei were stained with DAPI (blue). Bar, 20 μ m. Five examples of progressively later stages of each mitotic stage are presented. The cell periphery is outlined in white. Selected nuclei of, from left to right, prophase, metaphase, anaphase, and telophase. Bar = 10 μ m. (b) Serum-starved ARPE-19 cells were treated with 10 ng/ml TGF- β 1 for different times. Cell lysates were prepared and the level of Aurora B protein was analyzed by western blot with a specific antibody. *Ctrl*, control

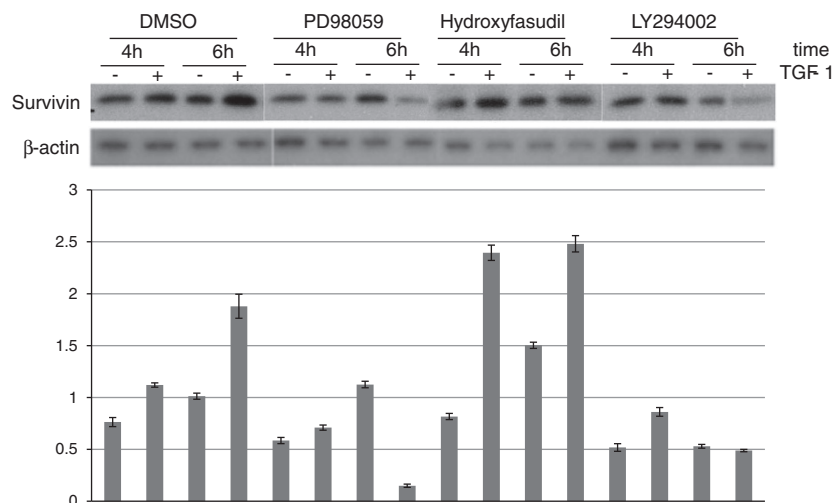


Figure 7 TGF- β 1 stimulates the upregulation of survivin via PI3K. Serum-starved ARPE-19 cells were pretreated for 1 h with DMSO vehicle or 10 μ M of MEK, RhoA or P13K inhibitors and then cultured in the absence or presence of 10 ng/ml TGF- β 1 for the indicated times. Cells lysates were prepared and the level of survivin protein was analyzed by western blotting with a specific antibody

can induce both cell survival and death under the same experimental conditions. It is likely that this differential response to TGF- β 1 according to the cell cycle phase is mediated by an interplay between TGF- β 1 signaling pathways

and proteins that regulate the cell cycle. TGF- β 1-induced cell cycle progression may be a prerequisite for cells to undergo EMT. As TGF- β 1-induced apoptosis and EMT mediated different effects during tumor progression and embryonic

development, they may be mutually exclusive processes. TGF- β -induced EMT leads to migration and invasion of local epithelial cells. These cells evade apoptosis, and this process is important for organogenesis and tumor metastasis. The fundamental role of TGF- β signaling in these cells may be EMT induction, not growth arrest.

Rb phosphorylation and the induction of cdc2 in response to TGF- β 1 were reduced following survivin depletion (Figure 3). This suggests that survivin may regulate the cell cycle and thereby stimulate cells to undergo EMT, rather than apoptosis, in response to TGF- β 1. Increasing evidence indicates that the cell cycle state influences cellular responses to extracellular stimuli. However, the ability of the same stimulus to induce two distinct cellular responses in the same cells, such as the induction of apoptosis or EMT by TGF- β 1, has not been studied in detail. Survivin regulates the G2/M phase of the cell cycle by associating with mitotic spindle microtubules and by directly inhibiting caspase-3 and -7.⁴² The current study showed that TGF- β 1 influences microtubule stability and stabilizes Aurora B during EMT (Figure 6). In figure 6a, we can see multiple mitotic processes, including prophase, metaphase, and telophase with survivin in TGF- β 1-treated cells. The ability of survivin to control microtubule dynamics during several phases of the cell cycle may have a dramatic impact on EMT. In the present study, we show that survivin has an important role in cell cycle regulation and affects microtubule stability during interphase. As shown in Figure 6a, during mitosis, a pool of survivin is localized within the chromosome passenger complex and regulates kinetochore–microtubule interactions; another pool of survivin is associated with spindle microtubules and regulates their stability.

The chromosomal passenger proteins, survivin and Aurora B, have an important auxiliary role in spindle checkpoint surveillance. Aurora B kinase activity regulates kinetochore–microtubule interactions and microtubule dynamics during mitosis. Of the chromosome passenger proteins, only Aurora B is a mitotic serine/threonine kinase. Aurora B is thought to have key roles in chromosome segregation, cytokinesis, and cancer development. Interestingly, Aurora B activity may require survivin, as Aurora B kinase activity was reduced following depletion of survivin (Figure 6b). Here, we show that survivin may not only operate in a complex with Aurora B, but also regulate the kinase activity of Aurora B.

We next identified the upstream signaling events responsible for the upregulation of survivin in response to TGF- β 1. This upregulation was abolished when cells were treated with chemical inhibitors of the MEK or PI3K signaling pathways, whereas inhibitors of Rho or ROCK had no effect (Figure 7).

In summary, our data demonstrate that survivin functions as a regulator of TGF- β 1-induced EMT throughout the cell cycle (Figure 8). TGF- β 1 can upregulate survivin expression via the PI3K pathway, and this increased level of survivin promotes cell cycle progression and microtubule stability, thereby inducing cells to undergo EMT and evade apoptosis.

An important conclusion of this study is that TGF- β 1-induced EMT is influenced by the cell cycle, and TGF- β 1 regulates cell cycle progression by upregulating survivin (Figure 8). Upregulated survivin by TGF- β 1 should act as a

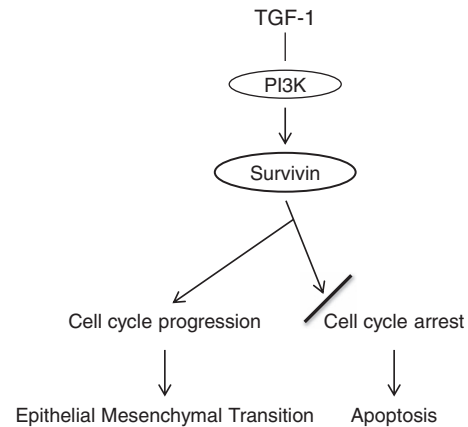


Figure 8 TGF- β 1 upregulates survivin to promote cell cycle progression and inhibit apoptosis during EMT. A mechanism is presented schematically to explain how cells undergo EMT or apoptosis in response to TGF- β 1

mitotic regulator and also activate Aurora B for cell cycle progression, so TGF- β 1 leads to multiple mitotic defects and cell apoptosis when survivin is absent.

Our data indicate that TGF- β can promote different effects under the same experimental conditions. It is likely that the differential effects of TGF- β (induction of growth arrest/apoptosis and EMT) are not related to a particular phase of cancer development or embryogenesis, but rather they are influenced by the cellular context and the specific cell cycle state of an individual cell. The sensitivity of tumor cells to TGF- β is likely influenced by genetic alterations, such as gene mutations or deletion of the TGF- β receptor gene, and may also be influenced by cell cycle status. Cell differentiation, migration, or apoptosis in response to TGF- β during early embryogenesis may be regulated, at least in part, by the cell cycle stage. Therefore, in addition to specific components of the TGF- β signaling pathway, it may be important to consider cell cycle status when researching new clinical therapies, including cancer treatments. These findings provide new insight into the mechanism by which TGF- β induces apoptosis and EMT, and explain, in part, the reasons why TGF- β treatment can induce different cell fates under the same experimental conditions. The detailed mechanism by which survivin influences cell fate following TGF- β treatment requires further study in relation to cell cycle status and regulators, the chromosomal passenger complex with Aurora B, microtubule dynamics, and caspase activity.

Materials and Methods

Cell culture and treatments. ARPE-19 cells were obtained from the American Type Culture Collection (Rockville, MD, USA) and maintained at 37 °C in a 5% CO₂ in DMEM/F-12 medium supplemented with 10% fetal bovine serum (FBS) (Gibco-BRL, Invitrogen, Carlsbad, CA, USA) and antibiotics (Penicillin–Streptomycin solution, Gibco-BRL). Hep3B were cultured in minimal essential medium supplemented with 10% FBS and 1% Penicillin–Streptomycin at 37 °C in a 5% CO₂ incubator. Cells were grown to confluence and were deprived of serum for 12 h before use.

The effect of TGF- β 1 on cell phenotype was determined by adding recombinant TGF- β 1 (10 ng/ml) to growth-arrested cell monolayers at 70% confluency. Cells were imaged using phase contrast microscopy. To test the effect of inhibitors on particular signaling molecules, the inhibitors were added to cells 1 h before TGF- β 1. All experiments were performed in the absence of FBS.

Antibodies and reagents. Human recombinant TGF- β 1 was purchased from R&D Systems (Minneapolis, MN, USA). Specific inhibitors of MEK (PD98059), PI3K (LY294002), and Rho (Hydroxyfasudil) were obtained from Calbiochem (La Jolla, CA, USA). The antibodies used for western blot analysis and immunocytochemistry were as follows: anti-phospho-Rb and anti-survivin (Cell Signaling, Beverly, MA, USA); anti-cdc2, anti-cdk2 and anti-acetylated tubulin (Santa Cruz Biotechnology, Santa Cruz, CA, USA); anti-Aurora B (Abcam, Cambridge, UK); and anti-actin (Sigma-Aldrich, St. Louis, MO, USA). Rhodamine and Alexa-labeled phalloidin were purchased from Molecular Probes (Eugene, OR, USA). RhoA and Rac1 assay reagents were from the Upstate Biotechnology (Temecula, CA, USA).

Flow cytometry analysis. Cell cycle status was quantitatively determined by flow cytometry analysis. ARPE-19 cells (1×10^5 cells/ml) were cultured in the absence or presence of 10 ng/ml TGF- β 1 for 24 or 48 h. Cells were collected, washed with cold PBS, fixed in cold (-20°C) 100% ethanol, treated with DNase-free RNase, and stained with 50 $\mu\text{g/ml}$ of PI. The DNA content was analyzed using a FACScan flow cytometer to quantify the proportion of cells in each cell cycle phase (Becton Dickinson, San Jose, CA, USA), and 10 000 gated events were acquired per sample. Analysis of cell cycle distribution (including sub-G $_1$: apoptosis) was performed with FlowJo software (TreeStar, Ashland, OR, USA).

Cell synchronization. To synchronize cells at the G1/S boundary, the cells were cultured in medium supplemented with 0.5 mM hydroxyurea for 24 h. G2/M synchronization was achieved by culturing cells in medium supplemented with 0.5 $\mu\text{g/ml}$ nocodazole for 20 h. After being synchronized in G1/S or G2/M phase, the cells were treated with TGF- β 1 for 24 h in the presence of hydroxyurea or nocodazole, respectively, and the level of apoptosis was analyzed.

Binding and staining of Annexin V. Annexin V binding was analyzed using the Annexin V-FITC Detection Kit I (Pharmingen, San Diego, CA, USA) according to the manufacturer's instructions. Briefly, cells were incubated with or without 10 ng/ml TGF- β 1 for 24 and 48 h. Cells were collected, washed twice with cold PBS, centrifuged at 1500 r.p.m. for 5 min, and resuspended in $1 \times$ binding buffer at a concentration of 1×10^6 cells/ml. Then, 100 μl of the solution (1×10^5 cells) were transferred to a 5 ml culture tube, and 5 μl of Annexin V-FITC, and 5 μl of PI were added. Cells were gently vortex-mixed and incubated for 15 min at room temperature in the dark, after which 400 μl of $1 \times$ binding buffer were added to each tube. The samples were analyzed by FACScan flow cytometry (Becton Dickinson). For each sample, 10 000 ungated events were acquired. Annexin V $^+$ PI $^-$ cells represented the early apoptotic population and Annexin V $^+$ PI $^+$ cells represented either late apoptotic or secondary necrotic populations.

Cell proliferation assay. ARPE-19 cells were seeded onto a 96-well plate at a concentration of 10^5 cells per well and were cultured in the presence or absence of 10 ng/ml TGF- β 1. The culture media was replaced with DMEM containing 10% FBS. The CCK-8 reagent was added 24 or 48 h after the addition of TGF- β 1. Plates were incubated at 37°C for 4 h and absorbance was measured at a wavelength of 450 nm using a microplate reader.

RNA isolation and reverse transcriptase (RT)-PCR of survivin mRNA. Total messenger RNA was isolated using Trizol reagent (Invitrogen) according to the manufacturer's instructions. One microgram of total RNA was transcribed to cDNA using an avian myeloblastosis virus reverse transcription system (Promega, Madison, WI, USA). A 100-mg aliquot of the resulting cDNA was amplified in a RT-PCR with 25 ng of β -actin-specific primers or Survivin-specific primers. PCR products were separated on a 1.2% agarose gel. Primers were as follows: Survivin forward primer, 5'-GGACCCGCTAAGAGGGCGTGC-3'; survivin reverse primer, 5'-AATGTAGAGATGCGGTGGTCCCT-3'; β -actin forward primer, 5'-GACGGGGTACCCACACTGTGCCCATCTA-3'; β -actin reverse primer, 5'-CTAGAAGCATTTGCGGTGGACGATGGAGG-3'.

Western blot analysis. Cells cultured in the absence or presence of TGF- β 1 in 6-cm dishes were scraped into 300- μl ice-cold lysis buffer (50 mM Tris-HCl (pH 7.4), 150 mM NaCl, 1 mM EDTA, 1% NP-40, 0.5% Na-deoxycholate, and 20 $\mu\text{l/ml}$ protease inhibitor cocktail (Pharmingen BD Biosciences, San Jose, CA, USA)). Samples were clarified by centrifugation at 13 000 r.p.m. for 5 min at 4°C and boiled for 5 min with Laemmli sample buffer containing 100 mM NaF. Protein concentrations were determined using the Bradford method

(Bio-Rad Laboratories, Richmond, CA, USA). Equivalent protein amounts were separated on 10% SDS-polyacrylamide gels and transferred to Immobilon-P polyvinylidene fluoride membranes (Millipore Corporation, Bedford, MA, USA). Blots were incubated with specific primary antibodies, and antigen-specific signals were detected using horseradish peroxidase-conjugated secondary antibodies and visualized using chemiluminescence (Pierce, Rockford, IL, USA).

siRNA treatment. Silencer pre-designed siRNA was used to specifically silence the *survivin* gene (Ambion, Austin, TX, USA). The targeted oligonucleotides sequences were: 5'-GGCAGUGUCCUUUUGCUAtt-3' (sense) and 5'-UAGCAAAGGGACACUGCCtt-3' (antisense). Silencer negative control no. 1 siRNA (Ambion) was used as a negative control. siRNA was delivered to ARPE-19 cells using different doses of SiPORT reagent (Ambion) according to the manufacturer's recommended protocol. The reduction in *survivin* gene expression following the treatment with survivin siRNA was measured by RT-PCR 48 h after transfection.

Immunofluorescence microscopy and phalloidin staining. Cells were cultured in four-well multi-chamber slides (Invitrogen) in serum-free medium for 12 h and then treated with recombinant 10 ng/ml TGF- β 1 for up to 2 days. At various time points, cells were rinsed for 3 min in PBS, fixed in 5% paraformaldehyde for 30 min, and permeabilized with 0.2% Triton-X100 in PBS for 20 min. After blocking for 1 h with 1% BSA/PBS, the cells were incubated with primary antibodies for 12 h at 4°C , and then incubated with FITC-conjugated or Alexa 568-labeled secondary antibodies (Molecular Probes). After being washed with PBS, the cells were mounted with FluorSave reagent (Calbiochem) and analyzed by confocal microscopy (Leica TCS 4D, Nussloch, Germany).

Statistical analysis. Statistical analysis was performed using the unpaired Student's *t*-test with a value of $P < 0.05$ considered to represent a significant difference.

Conflict of Interest

The authors declare no conflict of interest.

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