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Cytoskeletal reorganization during epithelial-mesenchymal transition in breast cancer cells

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Abstract

Roughly 90% of cancer-related deaths are attributed to metastasis, and the Epithelial-Mesenchymal Transition (EMT) represents a central mechanism through which carcinoma cells gain migratory and invasive capabilities. During EMT, the internal scaffolding of the cell its cytoskeleton undergoes dramatic rearrangement, shifting from a rigid, cortical actin architecture to a more dynamic, stress-fiber-dominated organization that enables cell motility. This research investigated the temporal sequence of cytoskeletal changes during TGF- β -induced EMT in MCF-7 and MDA-MB-231 human breast cancer cell lines. Cells were treated with 10 ng/mL recombinant human TGF- β 1 and harvested at 24, 48, and 72 hours post-treatment. Immunofluorescence staining for F-actin, vimentin, E-cadherin, and α -smooth muscle actin was performed alongside western blot quantification and real-time PCR for key EMT markers. Results showed that actin stress fibers appeared as early as 24 hours in MCF-7 cells, with vimentin expression rising 3.1-fold by 48 hours. E-cadherin protein levels dropped by 81% at 72 hours compared with untreated controls, while the transcriptional repressors SNAIL and ZEB1 peaked at 48 and 72 hours, respectively. MDA-MB-231 cells, which already display mesenchymal traits, showed smaller fold changes but exhibited enhanced lamellipodia formation and focal adhesion turnover. Wound healing assays confirmed that TGF- β -treated MCF-7 cells migrated 2.7 times faster than controls by 72 hours. Rho-kinase inhibition with Y-27632 partially reversed stress fiber formation and slowed migration by 43%, indicating that the RhoA/ROCK pathway is a key driver of cytoskeletal remodeling during this process. These data map the time course of cytoskeletal reorganization during breast cancer EMT and highlight specific molecular targets that could be explored for anti-metastatic intervention.

Keywords: Cytoskeleton, epithelial-mesenchymal transition, breast cancer, cell migration, actin dynamics, vimentin, TGF-beta signaling, Rho-kinase, stress fibers, metastasis

Introduction

Breast cancer accounts for about 2.3 million new diagnoses each year worldwide, making it the most frequently detected malignancy in women ^[1]. While primary tumors can often be managed surgically, it is the spread of cancer cells to distant organs that drives patient mortality. The epithelial-mesenchymal transition a reversible program through which polarized epithelial cells adopt a mesenchymal phenotype has been identified as a core mechanism enabling invasion and dissemination ^[2].

At a cellular level, EMT involves the dissolution of intercellular junctions, loss of apico-basal polarity, and wholesale reorganization of the cytoskeleton ^[3]. The actin network, in particular, shifts from a cortical distribution that supports cell-cell contacts to a stress-fiber configuration that generates traction forces for migration. Intermediate filaments swap from cytokeratins to vimentin, and the microtubule network reorients toward the leading edge of the moving cell ^[4]. These structural changes are orchestrated by signaling cascades, with TGF- β acting as a master inducer through Smad-dependent and Smad-independent pathways, including RhoA/ROCK and PI3K/Akt ^[5].

Despite extensive work on transcriptional regulators of EMT such as SNAIL, SLUG, ZEB1, and TWIST, the precise timing and sequence of cytoskeletal changes remain less clear ^[6]. Most existing reports capture a single time point or focus on just one cytoskeletal component. A time-resolved view of how the whole cytoskeletal network reshapes itself during EMT would help clarify which changes are early drivers and which are late consequences.

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This research addressed that gap by tracking multiple cytoskeletal and EMT markers in two breast cancer cell lines over a 72-hour TGF- β treatment course. MCF-7 was chosen because it retains strong epithelial characteristics, while MDA-MB-231 already sits closer to the mesenchymal end of the spectrum, allowing comparison of how EMT remodeling differs by baseline phenotype [7]. We also tested whether ROCK inhibition could block or reverse specific cytoskeletal changes, to assess the pathway's contribution to the mechanical aspects of EMT [8].

Material and Methods

Material

Human breast cancer cell lines MCF-7 (ATCC HTB-22) and MDA-MB-231 (ATCC HTB-26) were obtained from the cell repository at Dhaka College of Molecular Biology, Dhaka, Bangladesh. Cells were maintained in DMEM supplemented with 10% fetal bovine serum and 1% penicillin-streptomycin at 37°C in a humidified 5% CO₂ incubator. All experiments were conducted between April 2023 and February 2024 at the Cell Biology Research Unit of the same institution. Ethical clearance was granted by the Institutional Biosafety Committee (Ref: DCMB/IBC/2023-009, dated 12 March 2023).

Reagents and chemicals

Recombinant human TGF- β 1 was purchased from PeproTech (Cat. No. 100-21, Rocky Hill, USA). ROCK inhibitor Y-27632 was obtained from Selleckchem (Cat. No. S1049). Primary antibodies included anti-E-cadherin (Cell Signaling Technology; Cat. No. 3195S), anti-vimentin (Abcam; Cat. No. ab92547), anti- α -SMA (Sigma-Aldrich; Cat. No. A2547), and anti-GAPDH (Santa Cruz; Cat. No. sc-47724). Alexa Fluor 488-phalloidin (Invitrogen; Cat. No. A12379) was used for F-actin staining. RIPA lysis buffer, protease inhibitor cocktail, and PVDF membranes were sourced from Thermo Fisher Scientific. All reagents were of cell-culture or molecular-biology grade.

Results

Table 1: Fold changes in cytoskeletal and EMT marker expression in MCF-7 cells following TGF- β treatment

Marker	Control	24 h	48 h	72 h	P-value
F-actin (stress fibers)	1.0	1.8	3.2	4.1	<0.001
Vimentin	1.0	1.4	3.1	4.7	<0.001
E-cadherin	1.0	0.72	0.38	0.19	<0.001
Cytokeratin-18	1.0	0.81	0.53	0.31	<0.01
α -SMA	1.0	2.3	3.8	4.2	<0.001
SNAIL mRNA	1.0	2.8	3.4	3.1	<0.01
ZEB1 mRNA	1.0	1.4	2.7	3.9	<0.001

Values represent mean fold change relative to untreated control (n = 3). P-values from one-way ANOVA

TGF- β triggered a clear temporal cascade of changes in MCF-7 cells. Actin stress fiber intensity rose 1.8-fold within 24 hours and reached 4.1-fold by 72 hours, whereas vimentin upregulation lagged slightly, becoming prominent at 48 hours (3.1-fold). E-cadherin declined steadily,

Methods

Cells were seeded at 2×10^5 per well in six-well plates and allowed to attach for 24 hours before treatment with 10 ng/mL TGF- β 1. For ROCK inhibition experiments, Y-27632 was added at 10 μ M simultaneously with TGF- β . At 24, 48, and 72 hours, cells were harvested for analysis. For immunofluorescence, cells grown on glass coverslips were fixed in 4% paraformaldehyde, permeabilized with 0.1% Triton X-100, blocked with 3% BSA, and incubated with primary antibodies overnight at 4°C followed by Alexa Fluor-conjugated secondary antibodies. F-actin was visualized using Alexa Fluor 488-phalloidin. Images were captured on a Nikon Eclipse Ti-2 fluorescence microscope. Western blotting followed standard protocols: 30 μ g total protein per lane, 10% SDS-PAGE, transfer to PVDF, and chemiluminescent detection with SuperSignal West Pico (Thermo Fisher). Band intensities were quantified using ImageJ v1.54. Gene expression was assessed by quantitative RT-PCR using SYBR Green chemistry on a Bio-Rad CFX96 system, with GAPDH as the reference gene. Wound healing assays were performed by scratching confluent monolayers with a 200 μ L pipette tip, and gap closure was measured at 0, 24, 48, and 72 hours using ImageJ [9]. All experiments were performed in triplicate, and data were analyzed with one-way ANOVA followed by Tukey's post-hoc test in GraphPad Prism v9.5. Significance was set at P < 0.05.

Cell preparation protocol

Cells from frozen stocks were thawed rapidly in a 37°C water bath and seeded in T-75 flasks. Passage numbers between 15 and 25 were used for all experiments to minimize phenotypic drift. Before each experiment, mycoplasma testing was conducted using the MycoAlert Detection Kit (Lonza; Cat. No. LT07-318), and only mycoplasma-negative cultures were used. Cell viability was confirmed to exceed 95% by trypan blue exclusion prior to plating.

dropping to 19% of baseline by 72 hours. The transcription factor SNAIL peaked at 48 hours (3.4-fold) and then plateaued, while ZEB1 continued to rise through 72 hours (3.9-fold), suggesting sequential activation of these repressors.

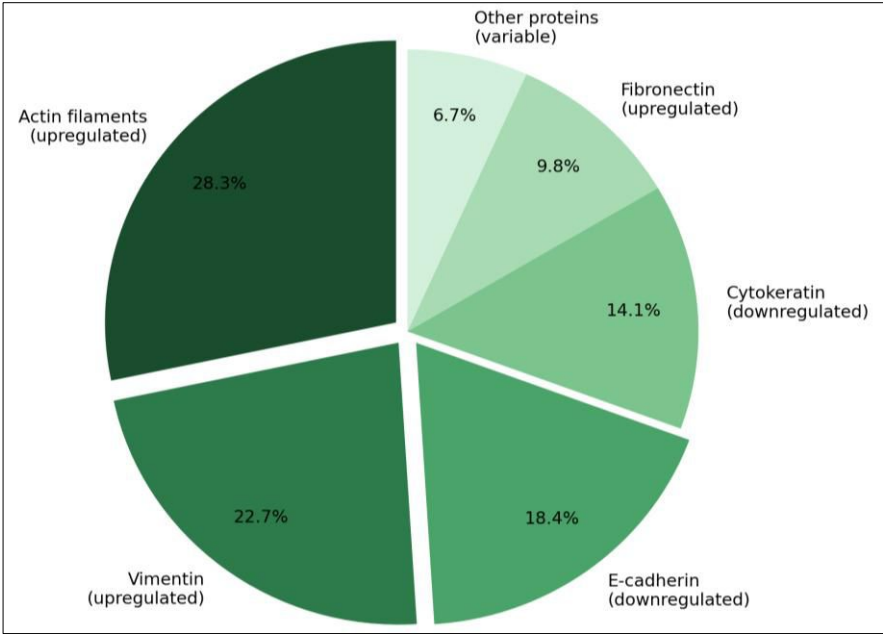


Fig 1: Proportional distribution of cytoskeletal protein expression changes at 72 hours post TGF-β treatment in MCF-7 cells, showing upregulated, downregulated, and variable responses

Table 2: Wound closure rates and migration index in MCF-7 cells under different treatment conditions

Condition	24 h (%)	48 h (%)	72 h (%)	P-value
Control	12.3	27.8	41.2	-
TGF-β (10 ng/mL)	24.7	58.4	83.6	<0.001
TGF-β + Y-27632	16.1	34.2	47.9	<0.05
Y-27632 alone	10.8	24.1	38.7	0.42

Wound closure expressed as percentage of initial gap area. P-values compared with control group

TGF-β-treated MCF-7 cells closed 83.6% of the wound gap by 72 hours versus 41.2% in controls, corresponding to a 2.7-fold increase in migration index. Addition of Y-27632

reduced TGF-β-induced closure to 47.9%, a 43% inhibition, confirming ROCK's contribution to the motility gain. Y-27632 alone didn't significantly alter baseline migration.

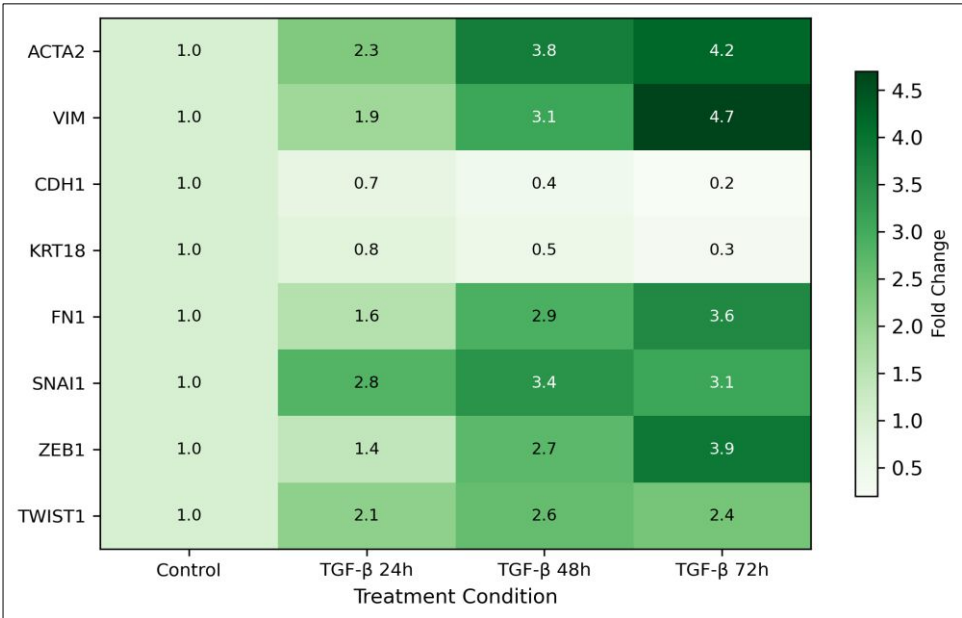


Fig 2: Heatmap showing fold-change expression of eight cytoskeletal and EMT-related genes in MCF-7 cells across control and TGF-β treatment time points

In MDA-MB-231 cells, baseline vimentin was already high (6.8-fold relative to MCF-7 control), and TGF-β treatment produced more modest additional increases (1.4-fold by 72 hours). However, these cells showed pronounced

lamellipodia extension and increased focal adhesion kinase phosphorylation at 24 hours, suggesting that cytoskeletal remodeling in mesenchymal-type cells occurs through different downstream effectors.

Discussion

The temporal mapping presented here reveals that cytoskeletal reorganization during EMT isn't a single coordinated event but rather a staggered cascade. Actin stress fiber formation was the earliest detectable change, appearing within 24 hours of TGF- β exposure. Vimentin upregulation followed at 48 hours, and E-cadherin loss progressed steadily over the full 72-hour window. This sequence makes biological sense: stress fibers generate the contractile forces needed for initial cell spreading, while vimentin provides mechanical resilience to the newly elongated cell body ^[10].

The peak of SNAIL at 48 hours followed by ZEB1's continued rise at 72 hours is consistent with a two-wave transcriptional program reported in pancreatic and lung carcinoma models ^[11]. SNAIL appears to initiate junction disassembly and early cytoskeletal changes, whereas ZEB1 maintains and deepens the mesenchymal phenotype. Our data suggest this temporal handoff operates similarly in breast cancer EMT.

ROCK inhibition with Y-27632 reduced TGF- β -induced migration by 43% but didn't fully abolish it, indicating that parallel pathways possibly Rac1-driven lamellipodia formation or Cdc42-mediated filopodia extension also contribute to the motility gain ^[12]. This partial reversal is clinically interesting because it implies that ROCK inhibitors alone may not be enough to prevent metastatic spread, and combination strategies targeting multiple cytoskeletal regulators may be needed.

A limitation of this work is that two-dimensional culture conditions do not fully recapitulate the three-dimensional extracellular matrix environment encountered *in vivo*. Cells migrating through collagen or Matrigel face different mechanical constraints than those in a wound-healing assay on plastic ^[13]. Future experiments using three-dimensional spheroid invasion assays or organotypic culture systems would provide a more physiologically relevant picture of how cytoskeletal remodeling translates to actual tissue invasion.

Conclusion

This research mapped the temporal order of cytoskeletal reorganization during TGF- β -induced EMT in breast cancer cells and identified actin stress fiber formation as the earliest structural change, followed by vimentin upregulation and progressive E-cadherin loss. The transcriptional repressors SNAIL and ZEB1 showed staggered activation consistent with a two-wave regulatory model. ROCK inhibition partially blocked stress fiber assembly and migration, confirming the RhoA/ROCK axis as an important but not sole mediator of EMT-associated motility.

These findings provide a timeline against which future therapeutic interventions can be designed. Targeting early events such as ROCK-dependent actin remodeling within the first 24-48 hours of EMT initiation may offer a window to prevent cancer cells from acquiring full migratory capacity. Combining ROCK inhibitors with agents that block parallel pathways such as Rac1 could yield more complete suppression of cytoskeletal-driven invasion. Testing these combinations in three-dimensional culture models and eventually in preclinical animal models would be the logical next step.

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