Research Square

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Context-dependent rewiring of autocrine TGFβ/RhoA/ROCK1 under complete vs deprived serum conditions in TNBC transduced cells

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Research Article

Keywords: Triple negative breast cancer, Transforming Growth Factor β, TGFβ, RhoA, TGFβ isoforms

Posted Date: September 15th, 2026

DOI: https://doi.org/10.21203/rs.3.rs-10936910/v1

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Additional Declarations: No competing interests reported.

Version of Record: A version of this preprint was published at Molecular Biology Reports on October 30th, 2026. See the published version at https://doi.org/10.1007/s11033-026-13061-6.

Abstract

Background

Triple-negative breast cancer is a challenging type of cancer due to its high recurrence rate, early metastasis, and resistance to therapies. Understanding the key regulatory mechanism to the mentioned oncogenic features is crucial. One such factor being studied is transforming growth factor beta, which has dual roles in promoting tumour growth and controlling cell proliferation. Therefore, this study aims to observe the differential of downstream RhoA/ROCK1 changes and proliferative outcomes in established TNBC cell lines.

Methodology

: MDA-MB-231 cells were transduced with lentivirus vectors harboring shRNA targeting different TGFβ isoforms: control cells transduced with non-targeting shRNA, shTGFβ1, shTGFβ2 and shTGFβ3 respectively. Transduction efficiency and target genes were assessed through RT-qPCR and cell proliferation was assessed using colony forming assay. Clinical relevance of RhoA-ROCK1was determined using the KM plotter data mining.

Results

The data showed an inverse TGFβ regulation between complete media (CM) and serum-derived (SR) condition. The interference of baseline growth factor in the complete media condition shown enhanced recovery of cell clonogenic capacity amidst the depletion of TGFβ isoforms. A more stable observation was seen in the SR condition with downregulation of RhoA-ROCK1 expression and reduction of cell survival upon TGFβ isoform depletion. However, compensory responses were observed in depleted shTGFβ2 and shTGFβ3 cells, whereby induction of clonogenic capacity and the recovery of TGFβ3 were observed respectively.

Conclusion

Serum-derived condition approach provides a consistent readout to measure TGFβ. This provides a better overview of the TGFβ/RhoA/ROCK1 activation axis in TNBC cells. Extended assessments are warranted to fully validate the interaction between this non-canonical network in TNBC, which may be clinically relevant and serves as a new potential therapeutic strategy in TNBC.

INTRODUCTION

Triple-negative breast cancer (TNBC) is a subtype of breast cancer that characterized by the absence of estrogen receptor (ER), progesterone receptor (PR), and low expression of human epidermal growth

factor 2 receptor (HER2) (Orrantia-Borunda et al. 2022). It represents 10–15% of overall breast cancer with higher prevalence among women younger than age 40, black women, and those with a BRCA1 mutation, while it accounts for approximately 5% of all cancer-related deaths (1,2).

TNBCs are highly aggressive and clinically complex subtype of breast cancer. They are often characterized by metastases, a poor prognosis, high rates of recurrence, and resistance to standard endocrine treatment (2–4). They are often characterized by high histologic grade, indicated by more abnormal cells under a microscope with an increased proliferation rate (5,6). A significant proportion of TNBC tumours belong to the basal-like subtype, characterized by the expression of genes associated with basal cells in the breast (7). High-grade tumours and basal-like tumours tend to be more aggressive and have a poorer prognosis (6,8). TNBC represents a heterogeneous group of tumours with TNBC represents a diverse collection of malignancies comprising numerous subtypes distinguished by unique genetic variations and signaling pathways (5). This heterogeneity also contributes to the diverse responses to treatments and challenges in developing targeted therapies (5,7,9). Because of the lack of receptors, TNBC is resistant to hormone-targeted therapies (e.g., tamoxifen, aromatase inhibitors) and HER2-targeted therapies (e.g., trastuzumab) (10,11). Chemotherapy is the mainstay treatment for TNBC patients. However, TNBC tumours have been associated with a higher risk of tumour recurrence, leading to suboptimal response rates and treatment failure (11).

The molecular profile of TNBC is complex, hence understanding the signaling pathways that drive its progression is crucial for developing targeted therapeutic interventions. Transforming Growth Factor-Beta (TGFβ) signaling pathway is one of the classical commonly associated with driving oncogenic properties in cancer. TGF-β is a multifunctional growth factors that participate in cell proliferation, differentiation, apoptosis, migration, adhesion, extracellular matrix production, angiogenesis, and cellular immunity (12). TGFβ family has three isoforms that include TGFβ1, TGFβ2, and TGFβ3. Dysregulation of TGFβ signalling has been associated with various diseases, including cancer, fibrosis, autoimmune disorders, and developmental disorders (13,14). TGFβ has opposing effects on cancer depending on its stage. In early stage of cancer TGFβ enforces homeostasis and suppresses tumour progression through its tumour suppressive effects and effects on stroma (11,15,16). While in late stage of cancer, cancer cells use TGFβ to their advantage to promote tumour progression through its immunosuppressive action and epithelial mesenchymal transmission EMT induction (15,16).

TGFβ can induce the process of EMT in TNBC cells, leading to increased invasion and migration capabilities, this process is associated with tumour metastasis and resistance to therapy (17,18). TGF-β has immunosuppressive effects, besides dampening the immune response against tumor cells through inhibiting the activity of immune cells and reducing the anti-tumour immune response, it has been demonstrated that TGF-β suppresses the antitumor function of genetically engineered immune cells against TNBC in preclinical models (19,20). Additionally, the TGF-β signalling pathway is associated with metastases and poor overall survival in TNBC patients (21).

TGFβ signaling pathway are involved in multiple activations; canonical SMAD and non-canonical pathway activation. One of the common non-canonical pathways is involving the RhoA activation. RhoA (Ras homolog family member A) and ROCK1 (Rho-associated protein kinase 1) play crucial roles in the Rho signaling pathway, which is responsible for controlling a range of cellular processes, particularly those associated with the actin cytoskeleton (22). RhoA is a member of the Rho family of GTP-binding proteins, functioning as a molecular switch that transitions between an inactive GDP-bound state and an active GTP-bound state (23). Its activation significantly impacts actin cytoskeleton dynamics by modulating stress fiber and focal adhesion formation, consequently influencing cellular functions like cell shape, migration, and adhesion (24). ROCK1 is a serine/threonine kinase and a major downstream effector of RhoA (25). It is activated by the binding of GTP-bound RhoA (24). ROCK1 phosphorylates various substrates, including myosin light chain, leading to the reorganization of the actin cytoskeleton (25). This kinase is involved in the regulation of cell contractility, migration, and other processes dependent on actin-myosin interactions. Together, RhoA and ROCK1 form a signaling pathway that plays a crucial role in cellular functions such as cell motility, adhesion, and morphology (24). Dysregulation of this pathway has been implicated in various diseases, including cancer, where aberrant RhoA/ROCK1 signaling can contribute to tumor progression and metastasis (23).

Given its dual role in TNBC, TGF-β has become a potential target for therapeutic intervention (26,27). Researchers are investigating drugs that can block TGF-β signalling pathways (small molecule TGF-β inhibitors) to inhibit its tumour promoting effects. Utilizing the small molecule inhibitors is actively explored at clinical trials, however, the molecular mechanism is not fully understood in TNBC. Though this study is reversing back to the fundamental, we aimed to investigate the downstream TGF-β non-canonical RhoA-ROCK1 activation and proliferative effects of each isoform in the MDA-MB-231 TNBC cell line.

METHODOLOGY

Cell culture

Cell culture procedures were adhered to rigorous aseptic techniques, conducted within a UV-treated biosafety cabinet to minimize the risk of contamination. To ensure a sterile working environment, surfaces were thoroughly decontaminated using 70% (v/v) ethanol. The cell lines under investigation were cultivated in various vessels, including T25 and T75 flasks, 6-well, 24-well, and P60 culture plates, and the culture medium was DMEM with 10% FBS and 1% Antibiotic-Antimycotic. These cultures were maintained in incubators set to a controlled atmosphere of 5% carbon dioxide (CO2) at 37°C, within a humidified environment. Daily monitoring of the cell cultures was undertaken and passaging and seeding occurred when cells reached 80–90% confluency. The cells were seeded and harvested in two conditions; in 10% FBS complete media (CM) and 2% FBS serum-reduced (SR) conditions.

Cells transduction

MDA-MB-231 cell line used in this study was obtained from ATCC. Cell transduction with pLKO-puro lentiviral vectors obtained from Sigma-Aldrich MISSION® shRNA (Table S1) was carried out. The lentiviral vectors were specifically engineered with short hairpin RNA sequences designed to interfere with and effectively knockdown target genes. This targeted interference strategy was employed to modulate the expression of TGFβ1, TGFβ2, and TGFβ3 genes in three distinct parental cell lines. As a negative control, cells were transduced with a non-targeting shRNA (shNT) cloned vector. Cells undergone 1µg/ml of puromycin selection for transduced cells with inserts. Four genetically distinct cells were obtained: control cells transduced with non-targeting shRNA, TGFβ1, TGFβ2 and TGFβ3 downregulated cells transduced respectively. Subsequently, transduction efficiency and downstream genes were assessed through RqPCR, and cell survival was assessed using colony forming assay.

RT-qPCR

Cells were seeded onto P60 plates overnight to reach 80–90% confluency. RNA was extracted utilizing the conventional phenol-chloroform extraction method (the full procedure is shown in Fig. 2.2). Subsequently, total RNA concentrations and purity were measured by using Thermo-scientific NANODROP 2000c. 2µg of total RNA was transcribed into cDNA according to the instructions of the High-Capacity cDNA Reverse Transcription Kit (Thermo Fisher Scientific, Lithuania (Cat.: 4368814), following the guidelines outlined in the provided manual. RT-qPCR reaction was conducted following the manufacturer protocol (QuantiNova SYBR Green RT-qPCR Kit, QIAGEN USA. Cat. 208352). Relative expression (RE) levels were calculated using the formula RE = 2^(-ΔΔCt), where ΔΔCt represents the normalized difference in threshold cycle (Ct) number between the control and transduced samples. Each Ct value was determined, and all samples were normalized to the corresponding expression levels of GAPDH and HPRT. The primers used are shown in Table S2.

Colony forming assay

The colony-forming assay was conducted to assess the survival capacity of cells. Cells were seeded at a density of 5×10^3 cells per well in 6-well plates and incubated for 6 days. Subsequently, the cells were washed, fixed with 100% methanol, and stained using 0.5% (w/v) crystal violet prepared in 20% (v/v) methanol. Plate images were captured using ChemiDoc Imaging Systems. The stained cells were then destained using 10% (v/v) acetic acid, and the absorbance A_{590nm} of dissolved stain was measured. The relative percentage was calculated for each transduced sample against the control sample.

Statistical analysis

Data obtained was analyzed using the GraphPad Prism software, version 9.0. The normality test was initially carried out to check the distribution of the data. Independent T-test or Mann-Whitney test was used to compare each transduced cells with the control. All the data were presented as mean ± SEM.

Significance was assigned at P-value < 0.05. The test was considered statistically significant if *P < 0.05, **P < 0.01, ***P < 0.001, ****P < 0.0001, ns; not significant.

KM plotter data mining

KM plotter (http://kmplot.com/analysis/) (28) data mining was carried out to assess the clinical relevance of RhoA and ROCK1 in TNBC protein expression data. Disease-free survival analysis was generated by selecting negative expressions of oestrogen, progesterone and low HER2 receptors. The impact size was determined by hazard ratios (HR), 95% confidence intervals (CIs), and statistical significance by log-rank p-values (p < 0.05).

Illustration Generation

Illustration in the manuscript was generated using Gemini Notebook.

RESULTS

RhoA-ROCK1 expressions in response to the downregulation of TGFβ isoforms

The knockdown efficiency of the target genes was determined by RT-qPCR compared to its control shNT cells. The results showed significant downregulation of TGFβ1 against its shNT control in complete media (CM) condition (Fig. 1A). Following this, significant reduction of RhoA (Fig. 1B) and ROCK1 (Fig. 1C) were observed upon depletion of TGFβ1. RhoA (Fig. 1E) expression was significantly reduced with ROCK1 (Fig. 1F) remain unchanged upon TGFβ1 knockdown in SR condition.

The TGFβ2-RhoA activation was observed in response to TGFβ2 knockdown in CM condition. Similar patterns were observed, with RhoA (Fig. 2B) and ROCK1 (Fig. 2C) were significantly downregulated upon TGFβ2 depletion. In SR condition, knocked down TGFβ2 (Fig. 2D) shown unchanged expressions of RhoA (Fig. 2E) but with elevated ROCK1 (Fig. 2F) expression observed.

Interestingly, upregulation of TGFβ3 was observed in the shTGFβ3-depleted cells (Fig. 3). To overcome this, a second puromycin selection step was carried out to obtain pure homogenous clones. However, the expression enhancement was still observed, possibly due to a compensation loop of TGFβ3, whereby the loss of TGFβ3 was compensated in the signaling pathway. In CM condition, RhoA expression was inconsistent (Fig. 3B) while ROCK1 was significantly downregulated (Fig. 3C) compared to the shNT control. Whilst in the SR condition, RhoA (Fig. 3E) and ROCK1 (Fig. 3F) expressions were significantly elevated upon TGFβ3 recovery.

MDA-MB-231 cell survival in response to TGFβ isoform downregulation

To assess the effect of TGFβ1 and TGFβ2 isoforms on the proliferation of MDA-MB-231 cell line, colony forming assay was carried out for both CM and SR conditions. In CM condition, each isoforms showed significant elevated colony forming ability compared to its shNT control (Fig. 4A and Fig. 4B). A trend of reduction of colony forming ability was seen by TGFβ1 and TGFβ3, but TGFβ2 showed significant elevation of colony formation (Fig. 4C and Fig. 4D).

Non-canonical RhoA-ROCK1 may be induced in the activation of TGFβ signaling in MDA-MB-231 cells

Kaplan-Meier generated from the KM Plotter database indicated RhoA and ROCK1 protein expression shown to have longer disease-free survival in TNBC patients with HR = 0.21, CI: 0.09–0.5 (p = 9.2e-05) and HR = 0.54, CI: 0.25–1.19 (p = 0.12) respectively (Fig. 5A and Fig. 5B). RhoA expression levels in the shTGFβ1 MDA-MB-231 (CM and SR) and shTGFβ2 (CM) are in concordance with the clinical outcome in TNBC.

DISCUSSION

Due to its complex involvement in the development of cancer, the differential roles of TGFβ isoforms, particularly TNBC, requires investigation. It has a context-dependent dual roles in the development and progression of cancer. During early stage, it delays the process of cellular transformation and prevents the development of cancer, however, in malignant mode, TGFβ takes a pivotal role in advancing tumor growth throughout later stages by primarily aiding epithelial to mesenchymal transition and causing immunosuppression (29–31).

Though this study only demonstrated cellular viability of the cells in the influenced of TGFβ isoforms, mainly to understand the distinct isoform roles or pathway activation in TNBC, these findings projected a new overview in the signaling activation in TNBC cell. The recovery of colony formation capabilities was observed in the CM condition (Fig. 6A), possibly influenced by the presence of baseline growth inducers such as TGFβ, IGF, FGF and EFG-like factors that controls the cell proliferation, survival, differentiation and EMT-like features. Other protein and hormone components present in the fetal bovine serum are also a possible contributing factor to elevated growth while each TGFβ isoform were concurrently depleted. To measure TGFβ roles, these limitations could be mitigated by conducting a serum-reduced condition as shown in this study or, inhibiting latent TGFβ in the fetal bovine serum to minimize the influence of basal TGFβ level in the media condition (32).

The SR condition exhibited a more stable and minimal basal interference as observed in cell survival and transcriptomic RhoA-ROCK1 activation. A hypothetical axis (Fig. 6B) was established referring to a trend of cell survival reduction being observed by the depletion of TGFβ1 with downregulation of RhoA and unchanged ROCK1 demonstrating a potential direct target axis in inhibiting cell survival in TNBC cells. An inverse reaction was seen in TGFβ2 whereby a possible TGFβ isoform compensation occurs with colony forming capability was increased. Interesting in TGFβ3 depleted cells orchestrating a potential compensation loop by RhoA-ROCK1upregulation to the recovery of TGFβ3 expression in this condition.

Whilst carrying out serum-reduced (SR) condition, it may potentially eliminate the loss of EGF basal function. Thus, this condition could ultimately induce background activation such as EMT-like features which hinders the suppression of growth in the absence of TGFβ1. Depleting single isoform may be a potential targetable strategy, there is a limitation, whereby ligand compensation may occur, potentially to be recruited by the TGFβ receptors to activate concurrent crosstalk of downstream signaling such as the RhoA/ROCK1, PI3K/Akt and MAPK/ERK pathways including the canonical SMAD-dependent axis (33–37).

This study highlighted a significant reduction in RhoA and ROCK1 expression following TGFβ1 downregulation, which suggests a potential dependence of RhoA and ROCK1 expression on TGFβ1 signaling. The stimulation of TGFβ1 in mesenchymal stem cells resulted in the activation of the RhoA/ROCK pathway (38). TGFβ1 was also shown to induce RhoA activation through GTPase pathway. Additionally, the study found that TGFβ1 can regulate EMT through RhoA-dependent pathways in non-transformed mouse mammary epithelial (39). Moreover, it has been shown that RhoA can be activated by TGF-β via either Smad-dependent or independent routes (40). This study also demonstrated a significant reduction in the expression levels of both RhoA and ROCK1 following TGFβ2 downregulation. This finding suggests a potential dependence of RhoA and ROCK1 expression on TGFβ2 signalling. RhoA can be activated by TGFβ via either Smad-dependent or independent routes (41).

Though the clinical relevance of the RhoA-ROCK1 and the recovery of TGFβ3 require further investigations, the concordant positive regulation of RhoA and ROCK1 were observed in the shTGFβ3, but cell survival capacity was not significantly hindered regardless the going through multiple cycles of antibiotic selection. This again suggesting a potential ligand-signaling crossroads to sustain oncogenic mode in the TNBC cell line. As such, Rho GTPase family, include RhoA, Rac1 and Cdc42 were reported to mediate stages of cell motility through modifications in the actin cytoskeleton. The cell adhesions are dependent to Rac1 actin-rich lamellipodium to adhere to the focal complexes, followed by maturation of cell adhesion in a RhoA-dependent manner (42). Interestingly, an in-silico study has shown an important role of the Rac1 signaling pathway activation in modulating a complementary role by each TGFβ isoforms via suppression of RhoA, thus further support a possible TGFβ ligand compensation in TNBC (43).

CONCLUSION

This study indicates a potential dependence of RhoA and ROCK1 activation in TGFβ signaling. Targeting single isoform may induce ligand compensation to activate downstream signaling mechanism. This study has limited in vitro assessments, therefore, in depth functional roles are warranted to fully understand the TGFβ-RhoA/ROCK1 signaling mechanism in TNBC. Suppression of all TGFβ isoform and recovery of individual isoform may deliver a more distinct characterization of the axis in TNBC. However, this study has shown a whole new perspective in the TGFβ-RhoA/ROCK1 worth further explored in a context-dependent manner. These findings underscore the complexity of TGFβ isoform signalling and its

impact on TNBC biology, highlighting the need for further research to elucidate underlying mechanisms and therapeutic implications.

Declarations

Ethics approval and consent to participate

Ethics approval was not required for this study. The source of samples were immortalised triple negative breast cancer cell line.

Consent for publication

Not applicable.

Availability of data and material

All data are included in the manuscript.

Competing interests

The authors declares there are no competing of interest.

Acknowledgement

Acknowledgement to Dr. Afreena Afiqah Azman and Associate Professor Dr. Nur Najmi Mohamad Anuar for their assistance in the laboratory logistics.

Funding

This study is funded by Geran Universiti Penyelidikan UKM (Grant code: GUP-2019-075) and Fundamental Research Grant Scheme (Grant code: FRGS/1/2025/SKK10/UKM/03/5).

Authors' contributions

EAMH; strategize in the structure, experimental designs, content and write up for each component of the manuscript.

EAEA; contributed in experiments, data analysis and introduction write up.

NNAD; contributed in the establishment of stable cells.

References

1. Adel, N. G. 2021. Current treatment landscape and emerging therapies for metastatic triple-negative breast cancer. *Am J Manag Care* 27(5 Suppl): S87-s96.

2. Almansour, N. M. 2022. Triple-Negative Breast Cancer: A Brief Review About Epidemiology, Risk Factors, Signaling Pathways, Treatment and Role of Artificial Intelligence. *Front Mol Biosci* 9: 836417.
3. American Cancer Society. 2023. Triple-negative Breast Cancer. https://www.cancer.org/cancer/types/breast-cancer/about/types-of-breast-cancer/triple-negative.html
4. Anders, C. K., Carey, L. A. & Burstein, H. J. 2021. ER/PR negative, HER2-negative (triple-negative) breast cancer. *UpToDate; Vora, SR, Ed.; Wolters Kluwer: Waltham, MA, USA*:
5. Bai, X., Ni, J., Beretov, J., Graham, P. & Li, Y. 2021. Triple-negative breast cancer therapeutic resistance: Where is the Achilles' heel? *Cancer Lett* 497: 100-111.
6. Batlle, E. & Massagué, J. 2019. Transforming Growth Factor-β Signaling in Immunity and Cancer. *Immunity* 50(4): 924-940.
7. Bhowmick, N. A., Ghiassi, M., Bakin, A., Aakre, M., Lundquist, C. A., Engel, M. E., Arteaga, C. L. & Moses, H. L. 2001. Transforming growth factor-beta1 mediates epithelial to mesenchymal transdifferentiation through a RhoA-dependent mechanism. *Mol Biol Cell* 12(1): 27-36.
8. Buyuk, B., Jin, S. & Ye, K. 2022. Epithelial-to-Mesenchymal Transition Signaling Pathways Responsible for Breast Cancer Metastasis. *Cell Mol Bioeng* 15(1): 1-13.
9. Davey, M. G., Hynes, S. O., Kerin, M. J., Miller, N. & Lowery, A. J. 2021. Ki-67 as a Prognostic Biomarker in Invasive Breast Cancer. *Cancers (Basel)* 13(17):
10. Derakhshan, F. & Reis-Filho, J. S. 2022. Pathogenesis of Triple-Negative Breast Cancer. *Annu Rev Pathol* 17: 181-204.
11. Eckenstaler, R., Hauke, M. & Benndorf, R. A. 2022. A current overview of RhoA, RhoB, and RhoC functions in vascular biology and pathology. *Biochemical Pharmacology* 206: 115321.
12. Glazer, E. S., Welsh, E., Pimiento, J. M., Teer, J. K. & Malafa, M. P. 2017. TGFβ1 overexpression is associated with improved survival and low tumor cell proliferation in patients with early-stage pancreatic ductal adenocarcinoma. *Oncotarget* 8(1): 999-1006.
13. Gómez-Gil, V. 2021. Therapeutic Implications of TGFβ in Cancer Treatment: A Systematic Review. *Cancers (Basel)* 13(3):
14. Guan, G., Cannon, R. D., Coates, D. E. & Mei, L. 2023. Effect of the Rho-Kinase/ROCK Signaling Pathway on Cytoskeleton Components. *Genes (Basel)* 14(2):
15. Hachim, M. Y., Hachim, I. Y., Dai, M., Ali, S. & Lebrun, J.-J. 2018. Differential expression of TGFβ isoforms in breast cancer highlights different roles during breast cancer progression. *Tumor Biology* 40(1): 101042831774825.
16. Haque, S. & Morris, J. C. 2017. Transforming growth factor-β: A therapeutic target for cancer. *Human Vaccines & Immunotherapeutics* 13(8): 1741-1750.
17. Jiao, Q., Wu, A., Shao, G., Peng, H., Wang, M., Ji, S., Liu, P. & Zhang, J. 2014. The latest progress in research on triple negative breast cancer (TNBC): risk factors, possible therapeutic targets and

prognostic markers. *J Thorac Dis* 6(9): 1329-1335.

18. Ko, E., Kim, D., Min, D. W., Kwon, S.-H. & Lee, J.-Y. 2021. Nrf2 regulates cell motility through RhoA–ROCK1 signalling in non-small-cell lung cancer cells. *Scientific Reports* 11(1): 1247.
19. Lebrun, J. J. 2012. The Dual Role of TGFβ in Human Cancer: From Tumor Suppression to Cancer Metastasis. *ISRN Mol Biol* 2012: 381428.
20. Li, X., Fan, Y., Tang, M., Li, H., Zhang, Y., Mi, J., Wang, Y., Zhao, M., Wang, Z. & Su, F. 2022. PLXDC1 Can Be a Biomarker for Poor Prognosis and Immune Evasion in Gastric Cancer. *J Inflamm Res* 15: 5439-5455.
21. Lu, T., Burdelya, L. G., Swiatkowski, S. M., Boiko, A. D., Howe, P. H., Stark, G. R. & Gudkov, A. V. 2004. Secreted transforming growth factor β2 activates NF-κB, blocks apoptosis, and is essential for the survival of some tumor cells. *Proceedings of the National Academy of Sciences* 101(18): 7112-7117.
22. Mahtani, R., Kittaneh, M., Kalinsky, K., Mamounas, E., Badve, S., Vogel, C., Lower, E., Schwartzberg, L. & Pegram, M. 2021. Advances in Therapeutic Approaches for Triple-Negative Breast Cancer. *Clin Breast Cancer* 21(5): 383-390.
23. Massagué, J. & Sheppard, D. 2023. TGF-β signaling in health and disease. *Cell* 186(19): 4007-4037.
24. Medina, M. A., Oza, G., Sharma, A., Arriaga, L. G., Hernández Hernández, J. M., Rotello, V. M. & Ramirez, J. T. 2020. Triple-Negative Breast Cancer: A Review of Conventional and Advanced Therapeutic Strategies. *Int J Environ Res Public Health* 17(6):
25. Orrantia-Borunda, E., Anchondo-Nuñez, P., Acuña-Aguilar, L. E., Gómez-Valles, F. O. & Ramírez-Valdespino, C. A. 2022. Subtypes of Breast Cancer. In (ed.). pp.31-42. Exon Publications.
26. Schmadeka, R., Harmon, B. E. & Singh, M. 2014. Triple-Negative Breast Carcinoma: Current and Emerging Concepts. *American Journal of Clinical Pathology* 141(4): 462-477.
27. Spiering, D. & Hodgson, L. 2011. Dynamics of the Rho-family small GTPases in actin regulation and motility. *Cell Adh Migr* 5(2): 170-180.
28. Stein, G. S., Lian, J. B., Montecino, M., Van Wijnen, J., Stein, J. L., Javed, A., Young, D. W., Hassan, M. Q. & Zaidi, S. K. 2008. Chapter 14 - Skeletal Gene Expression in Nuclear Microenvironments. In Bilezikian, J. P., Raisz, L. G. & Martin, T. J. (ed.). *Principles of Bone Biology (Third Edition),* pp.263-283. San Diego: Academic Press.
29. Stüber, T., Monjezi, R., Wallstabe, L., Kühnemundt, J., Nietzer, S. L., Dandekar, G., Wöckel, A., Einsele, H., Wischhusen, J. & Hudecek, M. 2020. Inhibition of TGF-β-receptor signaling augments the antitumor function of ROR1-specific CAR T-cells against triple-negative breast cancer. *J Immunother Cancer* 8(1):
30. Xu, T., Wu, M., Feng, J., Lin, X. & Gu, Z. 2012. RhoA/Rho kinase signaling regulates transforming growth factor-β1-induced chondrogenesis and actin organization of synovium-derived mesenchymal stem cells through interaction with the Smad pathway. *Int J Mol Med* 30(5): 1119-1125.
31. Xu, X., Zhang, L., He, X., Zhang, P., Sun, C., Xu, X., Lu, Y. & Li, F. 2018. TGF-β plays a vital role in triple-negative breast cancer (TNBC) drug-resistance through regulating stemness, EMT and apoptosis. *Biochem Biophys Res Commun* 502(1): 160-165.

32. Yang, R., Li, Y., Wang, H., Qin, T., Yin, X. & Ma, X. 2022. Therapeutic progress and challenges for triple negative breast cancer: targeted therapy and immunotherapy. *Mol Biomed* 3(1): 8.
33. Yang, Y., Ye, W. L., Zhang, R. N., He, X. S., Wang, J. R., Liu, Y. X., Wang, Y., Yang, X. M., Zhang, Y. J. & Gan, W. J. 2021. The Role of TGF-β Signaling Pathways in Cancer and Its Potential as a Therapeutic Target. *Evid Based Complement Alternat Med* 2021: 6675208.
34. Zhang, Y. E. 2009. Non-Smad pathways in TGF-β signaling. *Cell Research* 19(1): 128-139.

Figures

Figure 1

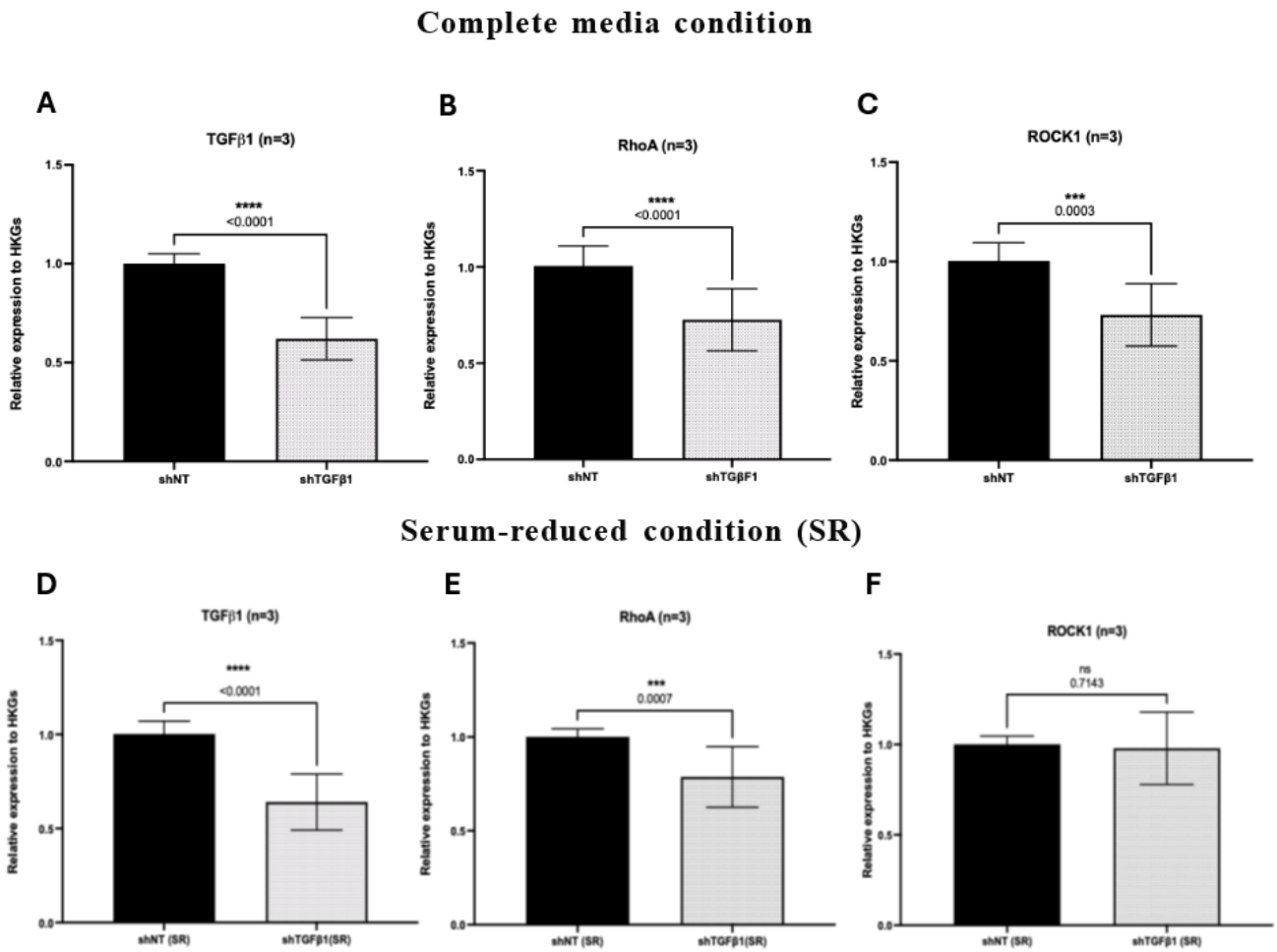


Figure 1

shRNA transduction efficiency and mRNA expression levels RhoA and ROCK1 using RT-qPCR. (A) Relative TGFβ1 expression in complete media condition shNT and shTGFβ1 cells. (B) Relative RhoA expression in complete media condition shNT and TGFβ1 cells. (C) Relative ROCK1 expression in complete media condition shNT and shTGFβ1 cells. (D) Relative TGFβ1 expression in serum-reduced

(SR) condition shNT and shTGFβ1 cells. (E) Relative RhoA expression in serum-reduced (SR) condition shNT and TGFβ1 cells. (F) Relative ROCK1 expression in serum-reduced (SR) condition shNT and shTGFβ1 cells. Data expressed in Mean ± SEM, * P <0.05, ** P <0.01, *** P<0.001, **** P <0.0001, ns; not significant.

Figure 2

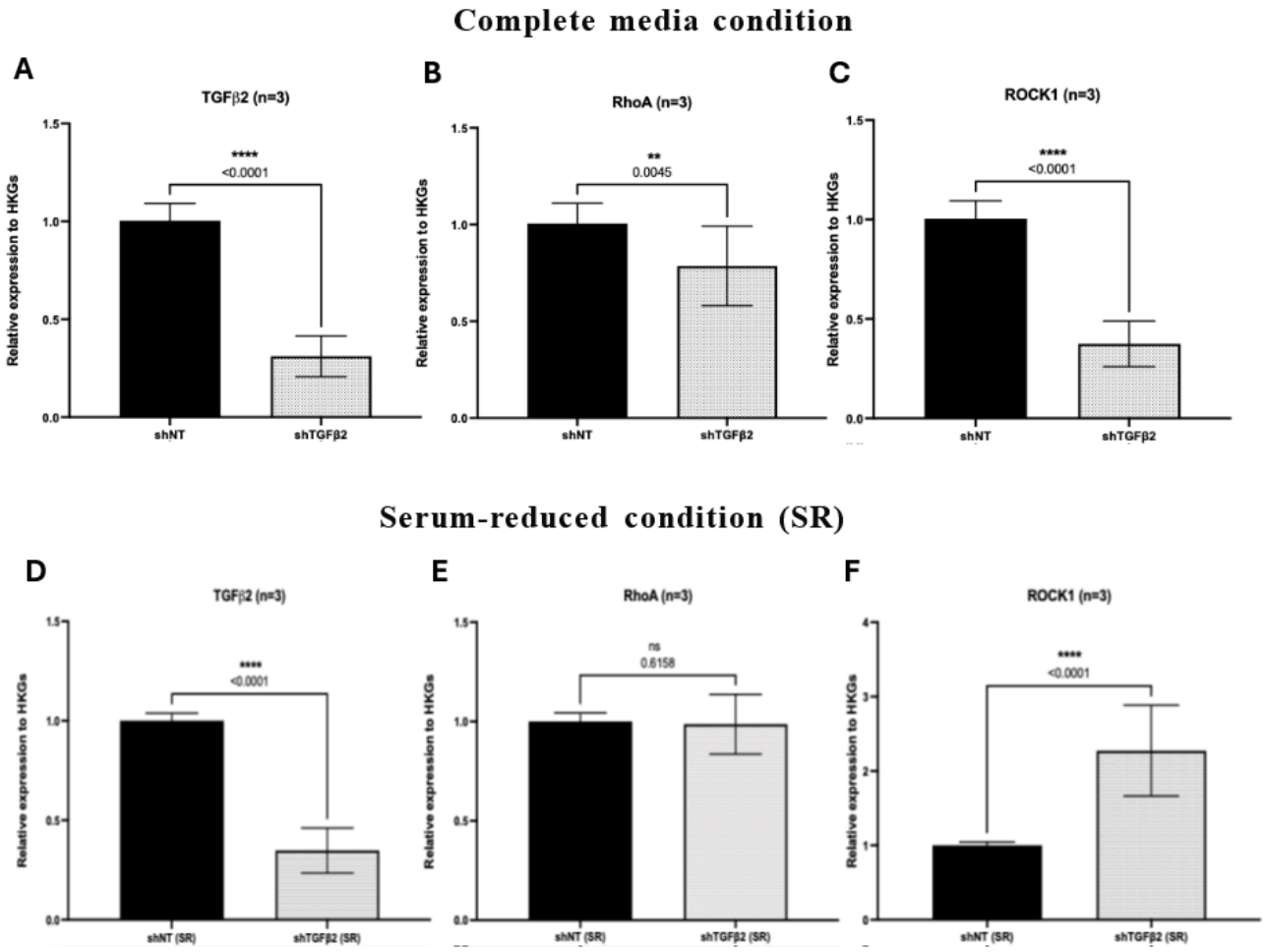


Figure 2

shRNA transduction efficiency and mRNA expression levels RhoA and ROCK1 using RT-qPCR. (A) Relative TGFβ2 expression in complete media condition shNT and shTGFβ2 cells. (B) Relative RhoA expression in complete media condition shNT and TGFβ2 cells. (C) Relative ROCK1 expression in complete media condition shNT and shTGFβ2 cells. (D) Relative TGFβ2 expression in serum-reduced (SR) condition shNT and shTGFβ2 cells. (E) Relative RhoA expression in serum-reduced (SR) condition shNT and TGFβ2 cells. (F) Relative ROCK1 expression in serum-reduced (SR) condition shNT and shTGFβ2 cells. Data expressed in Mean ± SEM, * P <0.05, ** P <0.01, *** P<0.001, **** P <0.0001, ns; not significant.

Figure 3

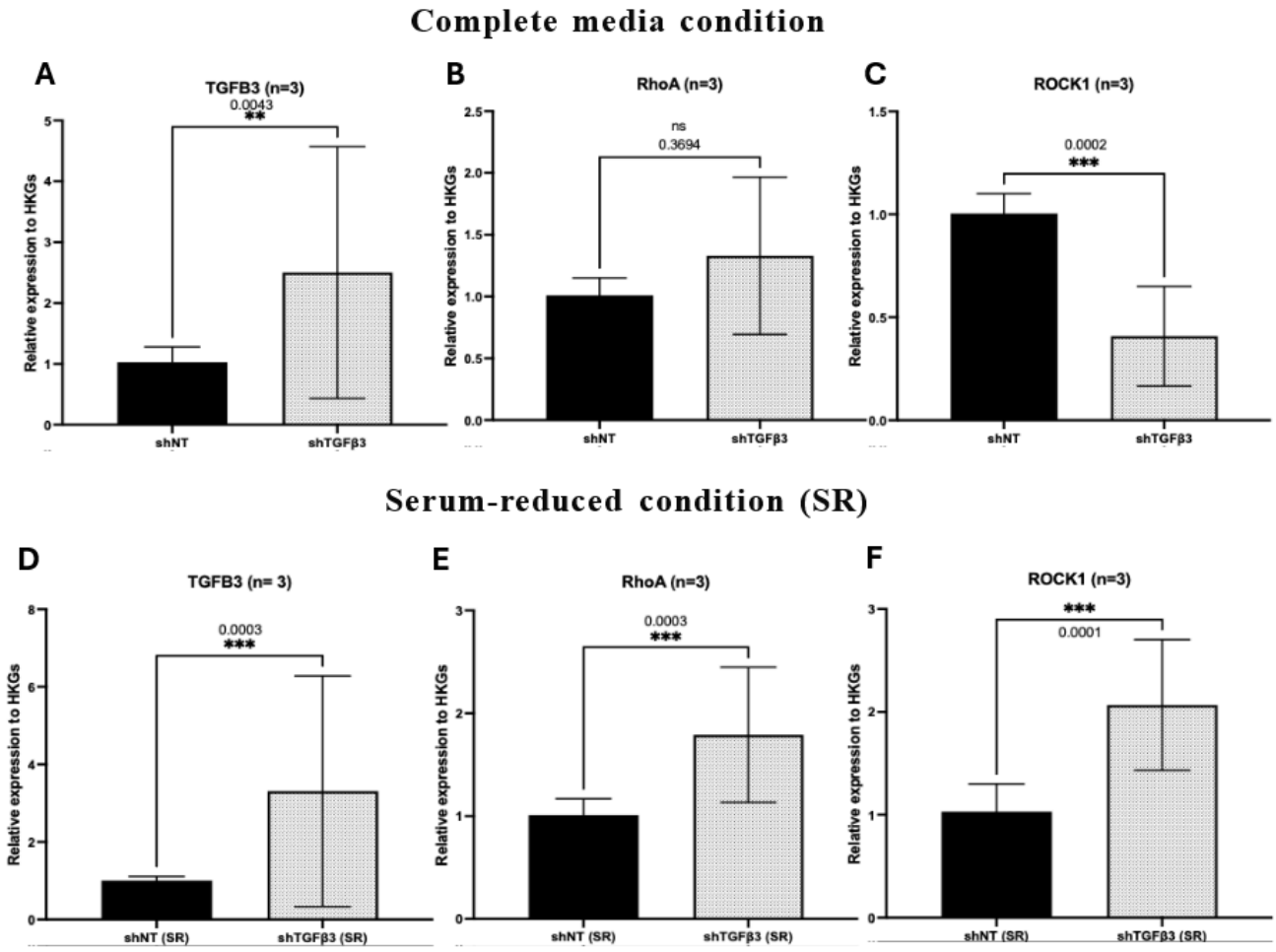


Figure 3

shRNA transduction efficiency and mRNA expression levels RhoA and ROCK1 using RT-qPCR. (A) Relative TGFβ3 expression in complete media condition shNT and shTGFβ3 cells. (B) Relative RhoA expression in complete media condition shNT and TGFβ3 cells. (C) Relative ROCK1 expression in complete media condition shNT and shTGFβ3 cells. (D) Relative TGFβ3 expression in serum-reduced (SR) condition shNT and shTGFβ3 cells. (E) Relative RhoA expression in serum-reduced (SR) condition shNT and TGFβ3 cells. (F) Relative ROCK1 expression in serum-reduced (SR) condition shNT and shTGFβ3 cells. Data expressed in Mean ± SEM, * P <0.05, ** P <0.01, *** P<0.001, **** P <0.0001, ns; not significant.

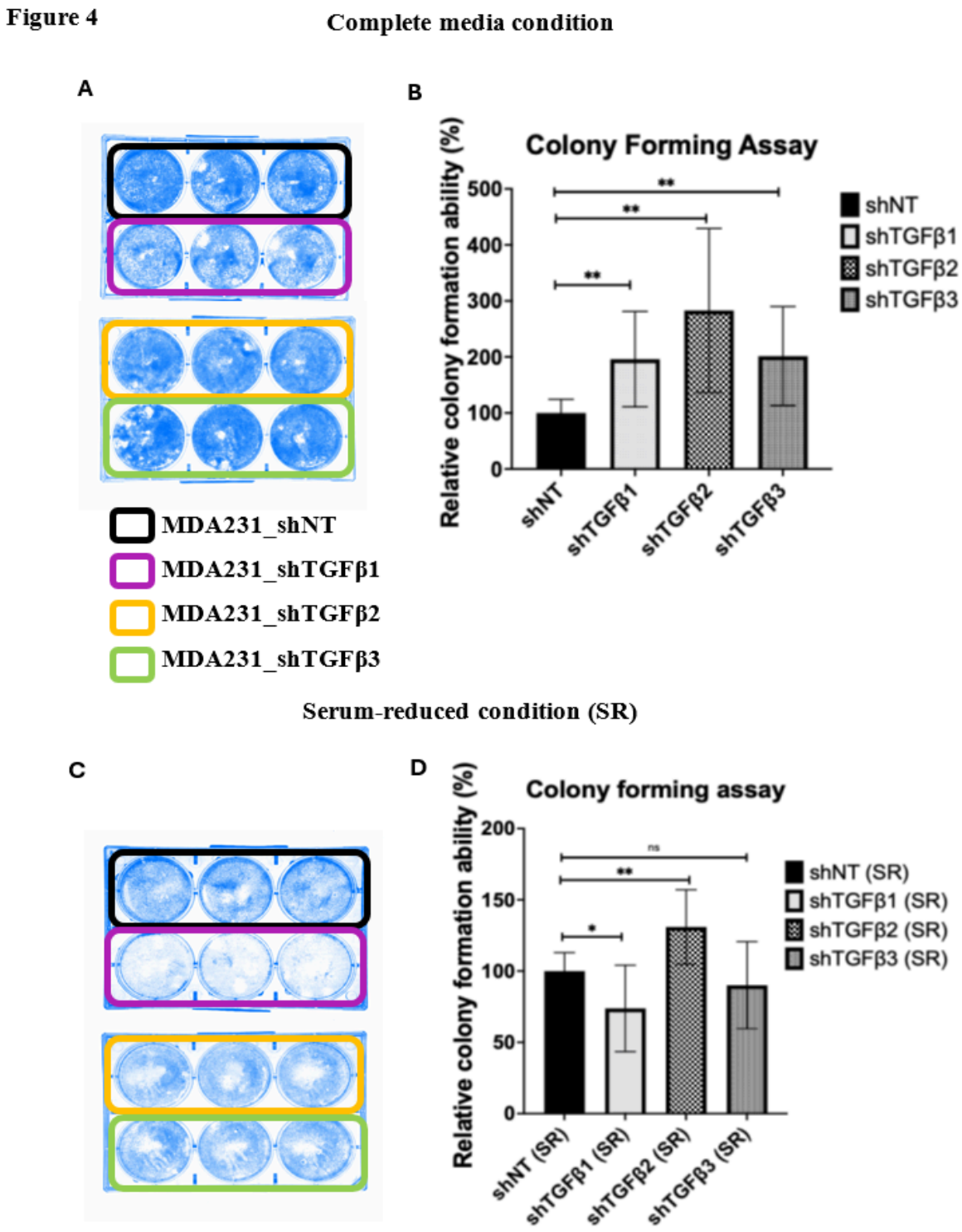


Figure 4

Colony forming assay to assess colony formation ability by TGFβ isoforms in MDA-MB-231 stable cell. (A) Colony forming assay of shNT, shTGFβ1, shTGFβ2 and shTGFβ3 in complete media condition. (B) Relative colony formation ability shNT and across TGFβ isoforms in complete media condition. (C) Relative TGFβ3 expression in shNT and shTGFβ3 cells in serum-reduced (SR) condition. (D) Relative

colony formation ability shNT and across TGFβ isoforms in serum-reduced (SR) condition. Data expressed in Mean ± SEM, * P <0.05, ** P <0.01, ns; not significant.

Figure 5

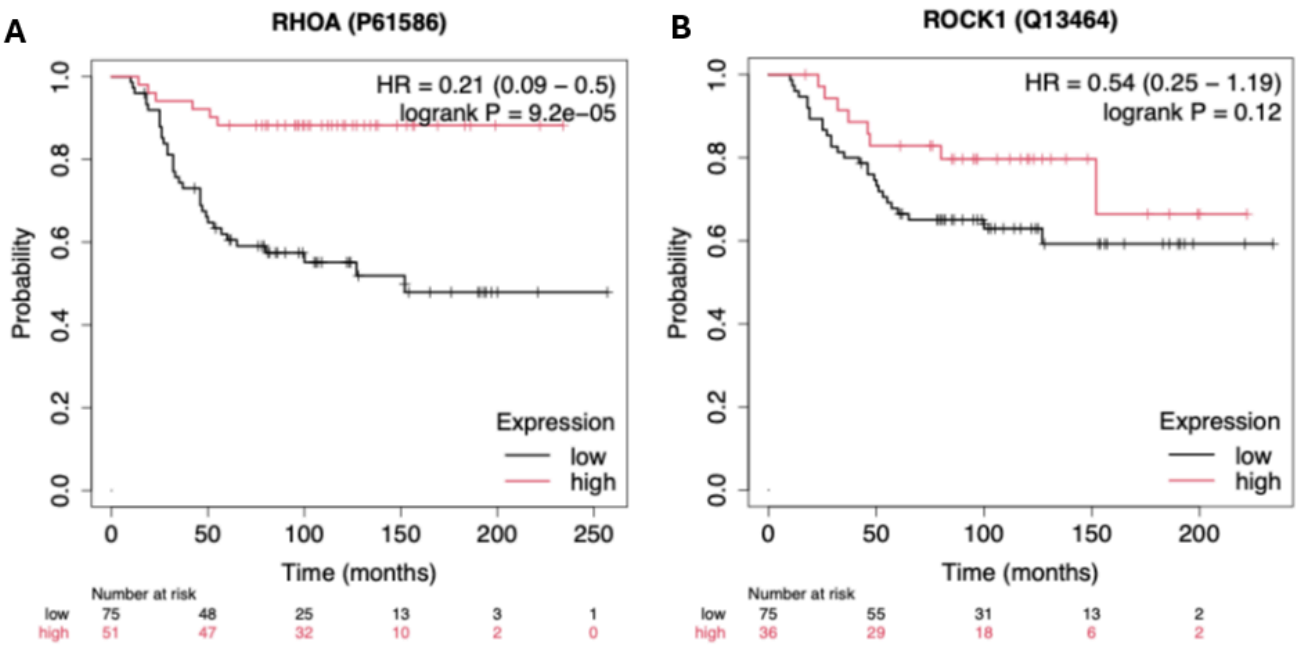


Figure 5

(A) A Kaplan-Meier generated from KM Plotter (Breast Cancer: Protein) indicating longer disease-free survival with RhoA protein expression in TNBC patients (B) A Kaplan-Meier generated from KM Plotter (Breast Cancer: Protein) indicating a pattern of longer disease-free survival with ROCK1 protein expression in TNBC patients.

Figure 6

A

Gene	RhoA (RqPCR)	ROCK1 (RqPCR)	Colony formation
TGFβ1 (CM)	▼****	▼***	▲*
TGFβ1 (SR)	▼***	= ns	▼*
TGFβ2 (CM)	▼**	▼****	▲**
TGFβ2 (SR)	= ns	▲****	▲*
TGFβ3 (CM)	▲ns	▼***	▲**
TGFβ3 (SR)	▲***	▲***	▼ns

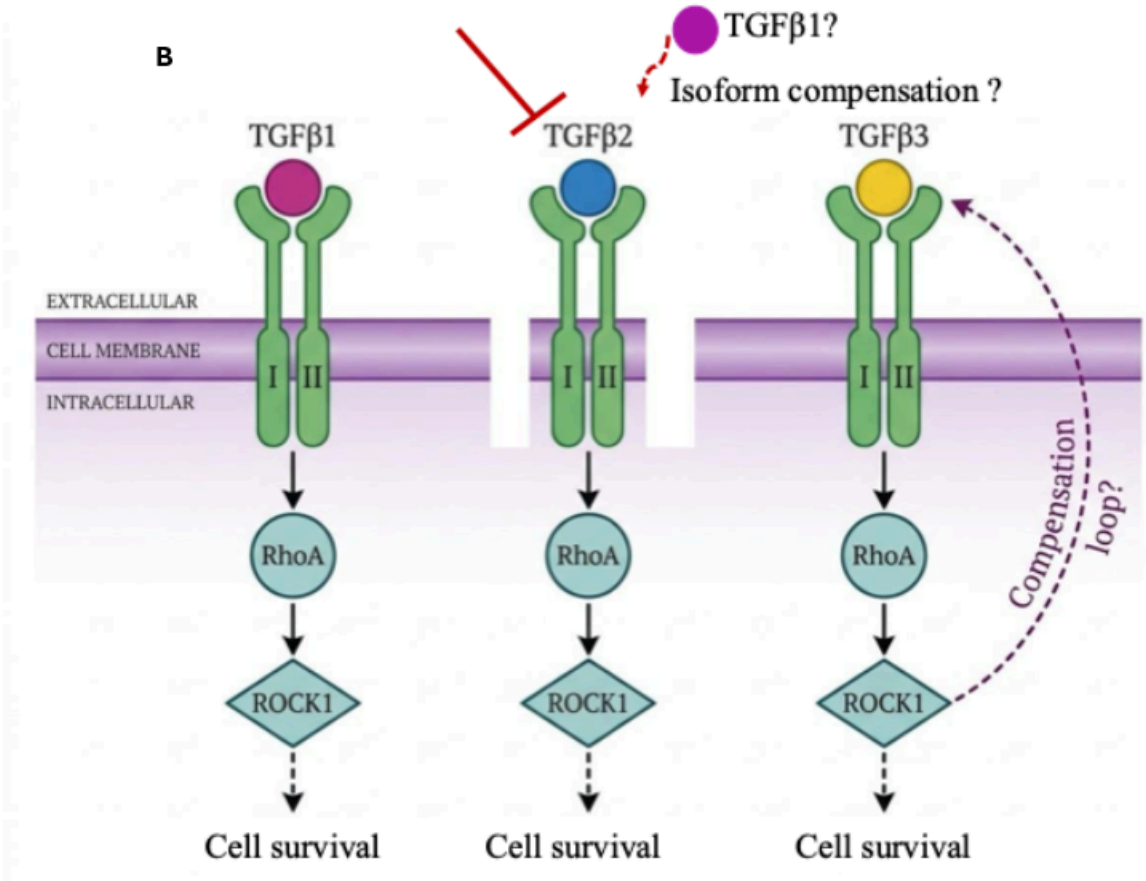


Figure 6

(A) A tabulated summary of RT-qPCR and colony formation assay (B) A hypothetical TGFβ-RhoA signaling pathway activation induced by a context-dependent manner and isoform compensation of ligand redundancy. Illustration was generated using Gemini Notebook.

Supplementary Files

This is a list of supplementary files associated with this preprint. Click to download.

- Supplementary05.09.2026.docx