Research Article

Fibroblast Modulation of Invasion and Chemoresistance in Triple-Negative Breast Cancer: Insights from a Two-Cell Organoid Model

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ABSTRACT

Objectives: Triple-negative breast cancer (TNBC) is a highly aggressive subtype of breast cancer characterized by the absence of hormonal receptors, which limits therapeutic options. Currently, chemotherapy remains the primary treatment, although resistance often develops over time. This study aimed to develop a co-culture organoid model to investigate the role of fibroblasts in TNBC cell invasion and chemoresistance.

Methods: A two-cell organoid model using the MDA-MB-231 cell line, a model cell for TNBC, and primary human foreskin fibroblasts (HDFs) was established. Their invasion and chemotherapy response were evaluated.

Results: Our data show that the fibroblasts facilitated invasion and chemoresistance. Hence, the important role of fibroblasts in modulating TNBC cell behavior was substantiated, as the contribution of fibroblasts in the TME was shown to promote enhancement of the invasion phenotype and decrease sensitivity to chemotherapy drugs.

Conclusion: This study highlights the significance of an organoid model in reproducing the tumor microenvironment (TME); hence, it provides evidence for the involvement of fibroblasts in the formation of TNBC. Therefore, the increased drug resistance and invasion observed in organoids with fibroblasts further advocate the relevance of targeting TME components when conceiving future therapeutic strategies for TNBC.

Keywords: Triple-negative breast cancer, Co-culture, Fibroblasts, Invasion, Drug resistance, Organoids

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Introduction

Triple-negative breast cancer (TNBC), defined by the absence of estrogen receptors (ER), progesterone receptor (PR), and human epidermal growth factor receptor 2 (HER2), accounts for 15–20 % of breast cancers and exhibits a notably poor prognosis due to its aggressive behavior and limited targeted therapy options (1, 2). Chemotherapy remains the standard systemic treatment; however, TNBC patients frequently develop chemoresistance and relapse, leading to high rates of metastatic progression. Recurrence often occurs months to years post-treatment, indicating intrinsic or acquired drug resistance in residual tumor cells (3, 4).

Emerging evidence highlights the tumor microenvironment (TME) as a key regulator of TNBC drug resistance, particularly via interactions with fibroblasts (5, 6). Fibroblasts secrete a diverse array of soluble factors—such as CXC motif chemokine 12 (CXCL12), interleukin 8 (IL-8), IL-6, transforming growth factor β (TGF-β), hepatocyte growth factor (HGF), epidermal growth factor (EGF), fibronectin, and extracellular matrix (ECM) proteins—that modulate tumor cell survival and therapy outcomes (7–9). For instance, IL-8 released by fibroblasts and macrophages enhances MDA-MB-231 cell proliferation and migration via CXCR1/2-mediated signaling, promoting chemoresistance and metastasis (10, 11).

Most in vitro studies utilize two-dimensional (2D) monoculture systems, which lack meaningful cell–cell and cell–ECM interactions present within tumors, while in vivo xenograft models, although more complex, present low throughput and poor control of microenvironmental variables (12). Consequently, these platforms inadequately mimic fibroblast-driven drug resistance phenomena and metastatic behavior in TNBC (13). Three-dimensional (3D) co-culture and organoid systems address these limitations by recapitulating tissue architecture and TME complexity (14). In particular, co-culture spheroids of TNBC cells with fibroblasts show increased resistance to paclitaxel, accompanied by activation of MAPK and PI3K/AKT signaling (15). Similarly, 3D co-cultures of MDA-MB-231 cells with primary or metastatic fibroblasts reveal enhanced invasion and ECM remodeling over extended culture periods (16). Indeed, fibroblast–TNBC interactions can significantly modulate chemotherapy response. A systematic co-culture drug screen indicated that fibroblasts from distinct tissue origins differentially influence TNBC sensitivity to cytotoxic and targeted agents (17). Some fibroblasts desensitize TNBC to drugs like palbociclib, while others sensitize cells to therapies such as etoposide (18). These context-dependent effects underscore the importance of physiologically relevant fibroblast-containing models.

While patient-derived organoids (PDOs) incorporating stromal cells offer high physiological relevance, they are costly and exhibit substantial donor variability (19). In contrast, cell line-derived 3D models allow precise experimental control, including co-culture with primary fibroblasts and modulation of cell ratios (20). The current study leverages this advantage by developing a two-cell organoid model comprising MDA-MB-231 cells and primary human fibroblasts. By systematically altering fibroblast proportions, this model replicates clinically relevant increases in chemoresistance and metastatic behavior, enabling mechanistic investigations of fibroblast-driven effects on TNBC. Our two-cell organoid approach offers a robust, controllable, and high-throughput platform that bridges the gap between simple 2D cultures and complex in vivo models. It provides a strategic tool to dissect fibroblast-mediated mechanisms underlying drug resistance and metastasis in TNBC, ultimately guiding future therapeutic interventions.

Material and Methods

Cell lines and chemicals

The human breast cancer cell line MDA-MB-231 and primary human foreskin fibroblasts (HFFs) were both obtained from the Stem Cell Technology Research Center (STRC, Iran). Dulbecco's Modified Eagle Medium (DMEM) high glucose, RPMI 1640 medium, Trypsin-EDTA (0.25%), Fetal Bovine Serum (FBS), and Penicillin/Streptomycin were all purchased from Gibco (USA). Phorbol 12-myristate 13-acetate (PMA), Collagen type I, Doxorubicin, and Giemsa staining solution were supplied by Sigma-Aldrich (USA). Hematoxylin solution was provided by Padtan Teb (Iran), and Eosin powder was purchased from Merck (Germany). For flow cytometry, fluorochrome-conjugated anti-CD11b antibody was obtained from BioLegend (USA). The ATP assay kit was purchased from Takara Bio (Japan).

2D cell culture

The cells were cultured in DMEM high-glucose supplemented with 10% fetal bovine serum (FBS) and 1% penicillin–streptomycin, and maintained at 37 °C in a 5% CO_2 incubator. The medium was replaced as needed.

2-cell organoid culture

For 2-cell organoid culture, three ratio combinations of MDA-MB-231 and HFF cells were cultured in a low-attachment 96-well plate. These combinations included HFF greater than MDA (F > M), HFF equal to MDA (F = M), and HFF less than MDA (F < M). Cultures were prepared at concentrations of 20,000 and 40,000 cells/mL in DMEM high-glucose supplemented with 10% FBS and 1% penicillin–streptomycin, and maintained at 37 °C in a 5% CO_2 incubator. Organoids were imaged

using an inverted microscope, and their growth kinetics were analyzed using GraphPad Prism 10 software.

Histological studies

The microtissues (organoids) were fixed in 10% formaldehyde. Then, they were dehydrated in a series of ethanol dilutions. Next, they were embedded in melted paraffin to prepare paraffin blocks that could be sectioned using a microtome. After sectioning, they were rehydrated in a series of ethanol dilutions to remove and cleared with xylene. Finally, staining with hematoxylin and eosin was performed.

Invasion test

To investigate cell invasion, organoids were cultured as described above. A 96-well plate was prepared 24 hours prior to the experiment by dissolving 4 mg of Type I collagen in 1 mL of PBS. Then, 100 µL of the collagen solution was added to the bottom of each well, and the plate was incubated overnight to allow gel polymerization.

After 24 hours, organoids were carefully removed from the non-adherent plates and transferred into the collagen-coated wells. Subsequently, 40 µL of culture medium was added to each well to cover the organoids. The plates were then incubated, and images were captured every 12 to 24 hours to monitor the invasion rate and process.

ATP assay

For the ATP assay, organoids were cultured as described in the previous sections. Four-day-old organoids were treated with 200 µL per well of doxorubicin solution at a concentration of 30 µM and incubated for 24 hours. After incubation, the treated organoids were collected and transferred into microtubes. The ATP assay was performed according to the standard protocol of the Takara kit, and luminescence was measured using a BioTek microplate reader.

Results

Establishment of a Two-Cell Organoid Model of Triple-Negative Breast Cancer

To develop an organoid model mimicking TNBC, the MDA-MB-231 cell line and primary HFF cells were used. Organoid cultures were performed at the ratios of F > M, F = M, and F < M. At all ratios, organoids reached sufficient density by day 2 (Fig. 1A), and their size increased during days 2–9, then stabilized, with no significant differences between the different cell ratios. In Fig. 1A, the growth curve for each cell ratio in the organoid culture is shown.

Fibroblast-Induced Lumen Reduction and Increased Mitotic Activity in TNBC Cells

To confirm organoid formation, sectioning and hematoxylin–eosin staining were performed on the microtissue paraffin blocks. Organoid formation criteria include the ability of cells to form specific structures of the desired organ and cellular heterogeneity. According to Fig. 1B, the generated organoids formed acini structures, which appear as lumens surrounded by cells. Nuclear polymorphism was also observed, mimicking intra-tumoral heterogeneity. The formed acini structures in the F > M organoids were fewer than those in the F = M organoids.

In Fig. 1-B, it is obvious that the extensive nuclear polymorphism in the microtissues and a low number of lumens formed. Also, it can be seen that nuclear polymorphism in organoids with a ratio of F = M is lower than in organoids with a ratio of F>M, which is an index of cancer progression that indicates the influence of fibroblast cells.

Enhanced Migration and Secondary Organoid Formation at Lower Fibroblast Ratios

To accomplish the size, structure, morphology, and the validity of organoid formation, they were examined for these properties. During the culture and parallel examination under the microscope, we observed that after the third day, the cells gradually separated from the mother organoid, migrated, and formed structures with a smaller size than the original organoid, but gradually grew and moved away from the original structure (Fig. 1-C). These structures can be called secondary organoids. Also, according to the graphs in Fig. 1-D and E, the size of these secondary organoids is almost the same in all ratios and no significant differences were seen (p-values are F<M vs. F=M = 0.8164, F<M vs. F>M= 0.8780 and F=M vs. F>M = 0.5225) but the number of these organoids is greater in the cell ratio F<M than in other ratios and there was a significant difference (p-values are F<M vs. F=M =<0.0001, F<M vs. F>M = <0.0001 and F=M vs. F>M = 0.1627) between F<M and F=M and also F>M. It is worth noting that this phenomenon was not observed in the case of luminal organoids made by MCF-7 cells, even after 14 days (Fig. 1-F). As a result, it seems that with an increase in the ratio of fibroblast cells, the rate of migration, movement, and formation of secondary organoids decreases.

Elevated Invasion with Higher Fibroblast Ratios

For examining the invasion ability of the cells in organoids in terms of the number of cells detached from the organoid over time, as shown in Fig. 2A–D, it seems that this number is highest in F<M organoids and lowest in F>M organoids (related p-values: F<M vs. F=M = 0.02885; F>M vs. F=M = 0.00579). In examining invasion by evaluating the distance traveled by invading cells (Fig. 2E), the results showed that it was longest for F>M and shortest for F<M.

Fibroblast-Induced Drug Resistance in TNBC Cells

To determine whether fibroblasts in the tumor

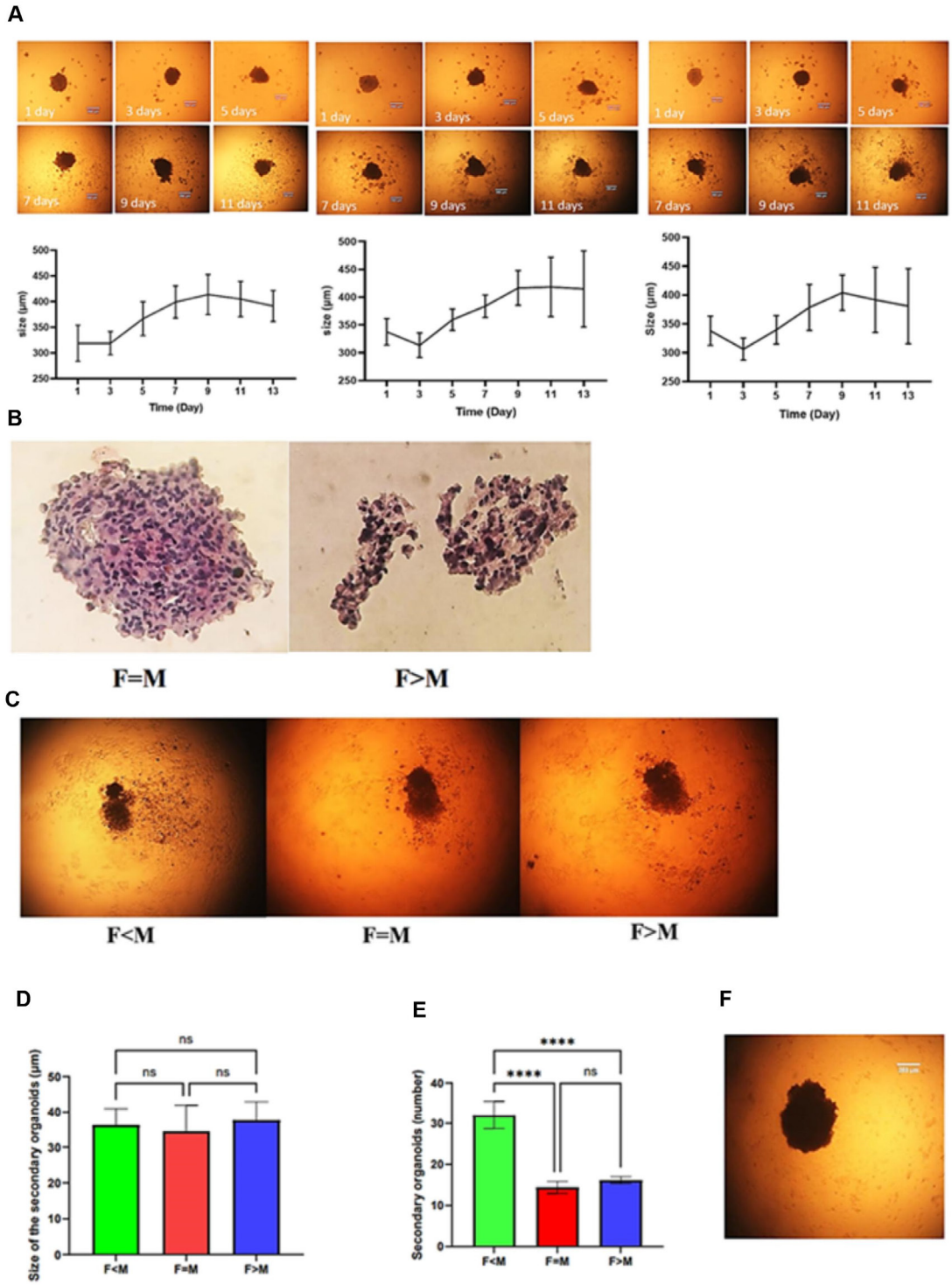

Figure 1. Morphological characterization and migration of organoids with varying fibroblast-to-cancer cell ratios. A, Organoids cultured at three ratios of fibroblast (F) to MDA-MB-231 (M): F>M, F= M, and F<M, imaged at days 1, 3, 5, 7, 9, and 11 (×10). Size measurements (µm) reveal differential growth trends across conditions. **B,** H&E staining of F=M organoids shows hallmark tumor features, including nuclear polymorphism (red), mitotic figures (yellow), and tubule/lumen formation (blue). Dense eosinophilic regions indicate high extracellular matrix (ECM) deposition. F>M organoids display enhanced nuclear atypia (red), rare tubule formation (blue), and highly dense ECM (green), consistent with higher tumor grade (×40). **C,** Migration and secondary organoid formation observed in F<M, F=M, and F>M conditions at days 14 and 20 (×4). Organoids formed with MCF-7 and fibroblasts (luminal A model) lacked secondary structures or notable migration, reflecting their low invasive potential. **D and E,** Quantification of the number and size (≥30 µm) of secondary organoids in different ratios at day 20. F>M organoids exhibited significantly increased migration and structure formation (**P****<0.0001**), indicating higher invasiveness. **F**, A luminal breast cancer organoid which does not show any secondary structures even after 14 days.

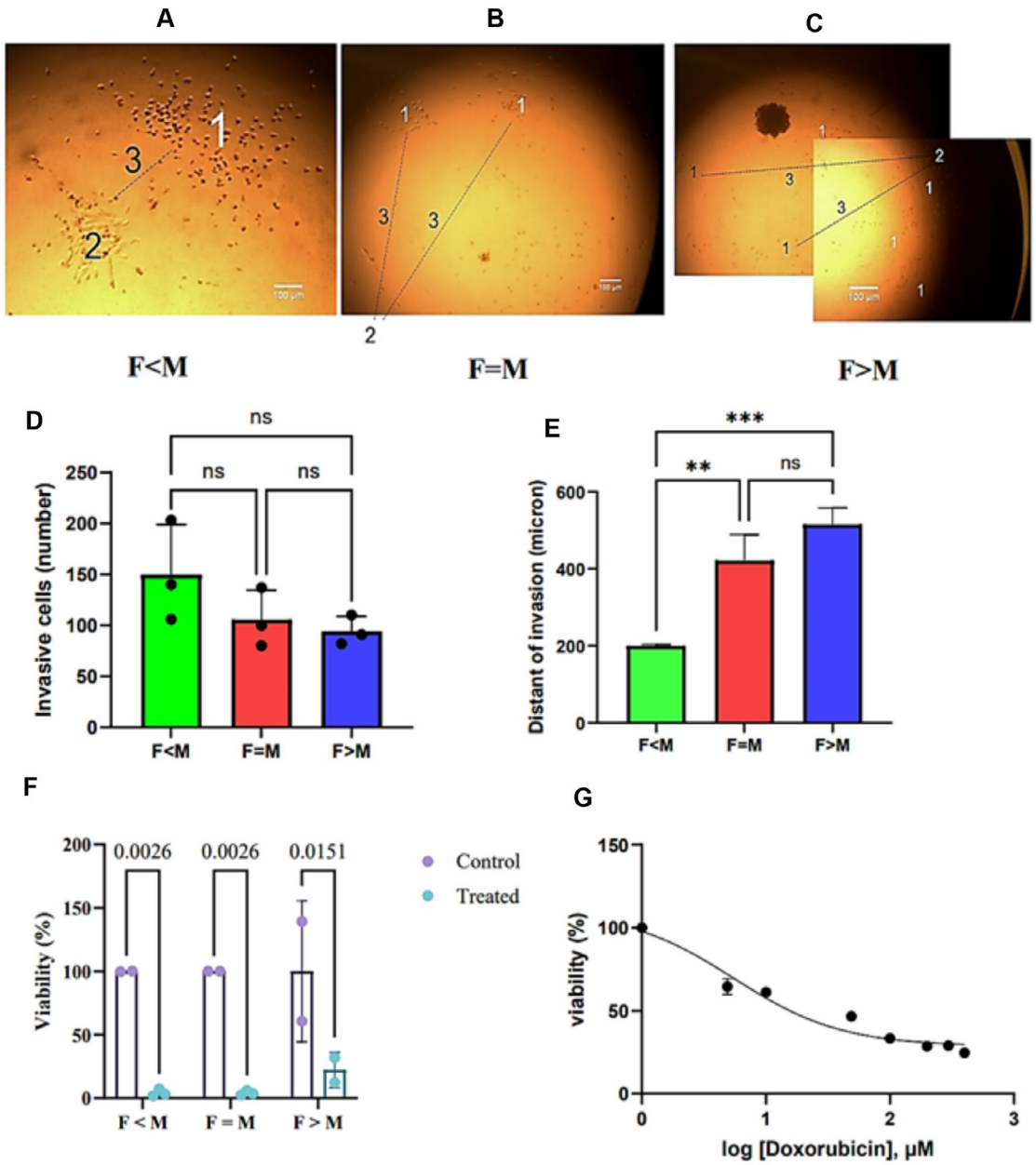

Figure 2. Metastatic behavior and chemoresistance in different organoids A, B and C, Invasion field of cells migrating from organoids seeded on type I collagen after 72 h, shown for F>M, F= M, and F< M conditions (×4). (1) Invasive cells, (2) initial organoid position and invasive footprints, and (3) migration distance are indicated. **D and E,** Quantification of the number and distance of invasion in each ratio after 72 h. Statistically significant differences were observed (**P-value** = 0.00579; *P* = 0.02885). **F and G**, drug resistance to chemotherapy drug, Doxorubicin, in the 24-h treatment of the organoids to IC50 dose (30 µM) of Doxorubicin.

microenvironment could act to promote chemoresistance (Fig. 2F) in TNBC cells against doxorubicin at its IC_{50} (30 µM) (Fig. 2G), a drug treatment was performed. The results showed that the drug response was nearly the same in the F<M and F=M groups (related p-values: F<M = 0.0026; F=M = 0.0026; F>M = 0.0151). However, the difference between the treated and control groups in the F>M organoids was less than in the other groups, indicating drug resistance in organoids with a higher ratio of fibroblasts.

Discussion

As cancer research advances, there is growing recognition of the need for reliable models that closely mimic human tumors. Traditional 2D cultures lack essential components of the tumor microenvironment

(TME), including stromal and immune cells, which are critical for intercellular communication and cancer-related signaling pathways. These interactions significantly influence tumor growth, invasion, and metastasis—features that 2D models fail to fully capture.

In this study, we developed a two-cell organoid model composed of MDA-MB-231 breast cancer cells and primary human foreskin fibroblasts (HFFs). Our goal was to establish a more physiologically relevant platform that includes both malignant and stromal components. By incorporating fibroblasts—key stromal cells involved in tumor progression through the secretion of growth factors and inflammatory cytokines—we aimed to create a model that better reflects the in vivo tumor microenvironment and provides more meaningful insights into tumor biology and therapeutic responses.

By developing our 3D two-cell organoid model, we confirmed that it faithfully mirrors key aspects of tumor biology, notably the structural and nuclear heterogeneity typical of higher-grade malignancies. Our experiment also demonstrated that the addition of fibroblasts to the organoid culture significantly altered cancer cell behavior, particularly their acinar formation capacity (Fig. 1). The co-cultured organoids appeared more compact and morphologically organized, with reduced lumen formation and increased nuclear atypia—features commonly associated with more advanced tumor grades. According to histological analysis, the former organoids are likely consistent with grades 2 to 3, based on the number and appearance of stained microtissue sections.

These findings further reinforce the physiological relevance of our model and its ability to recapitulate features of the natural tumor microenvironment. This observation is supported by previous literature: Falahi et al. demonstrated that their triple-negative breast cancer organoid model, comprising MDA-MB-231 cells co-cultured with human foreskin fibroblasts (HFFs), retained distinct intra-organoid diversity and growth dynamics akin to aggressive tumors. Importantly, they also reported that fibroblasts play a structural role in promoting cohesion among highly invasive cells like MDA-MB-231, which otherwise tend to remain dissociated. In this context, fibroblasts act as a "biological glue," enabling the formation of compact and morphologically organized organoid structures (20).

During organoid culture, we observed the formation of secondary structures indicative of cellular migration. This secondary organoid formation likely results from nutrient and oxygen depletion within the primary organoid. Migration is more pronounced in aggressive TNBC cells such as MDA-MB-231. Nutrient scarcity acts as a stressor, promoting invasive behavior. Increasing tumor grade leads to denser stroma and elevated fibroblast numbers, which enhance ECM production and stiffness. In our model, higher fibroblast content limited cell detachment and secondary organoid formation by creating a physical barrier. These findings align with Beslmüller et al. (2025), who showed that matrix stiffness regulates invasion in organoid systems, confirming the role of stromal stiffness in controlling tumor cell migration (21).

To assess whether enhanced migration reflects invasive behavior, we performed an invasion assay. Results (Fig. 2) showed that the group with higher fibroblast content (F>M) had fewer invasive cells but greater invasion distances. This suggests molecular changes in MDA-MB-231 cells driven by interactions with fibroblasts. Cells in organoids with lower fibroblast ratios detached more easily due to a less restrictive ECM but showed less invasion. Conversely, organoids with more fibroblasts had fewer exiting cells, yet these exhibited greater mobility and invasion, especially in the last 24 hours. These findings agree with Czekay et al. (2022), who reported that cancer-associated fibroblasts regulate ECM remodeling and signaling pathways that promote cancer cell invasion and metastasis (22).

To evaluate chemoresistance within the organoids, an ATP assay was performed. The results demonstrated that organoids with a higher fibroblast-to-cancer cell ratio exhibited significantly lower cell death compared to other groups, highlighting the crucial role of fibroblasts in promoting drug resistance (Fig. 2). Fibroblasts likely create a protective niche for malignant cells via paracrine signaling and secretion of survival-enhancing factors, thereby reducing cancer cells' susceptibility to chemotherapeutic agents. These findings are consistent with recent studies. Peng (2025) investigated fibroblast activation during chemotherapy in triple-negative breast cancer and showed that activated fibroblasts contribute to a microenvironment favoring tumor cell survival. Similarly, Miroshnychenko et al. (2023) reported that stroma-mediated breast cancer cell proliferation indirectly promotes chemoresistance by accelerating tumor recovery between chemotherapy cycles. Together, these studies reinforce the significant modulatory role of the tumor stroma in therapeutic response, underscoring the need to incorporate stromal components when developing effective treatments for TNBC (23, 24).

The model developed in this study consisted of two cell types with varying ratios, which were investigated in terms of metastatic behavior and drug response. It was found that increasing the ratio of fibroblast cells led to enhanced invasion and chemotherapy resistance. Each organoid ratio may represent a different stage of TNBC. This model, incorporating one of the most critical components of the tumor microenvironment, can be highly valuable and potentially useful in pharmaceutical, mechanistic, and histological studies. However, further research is needed to account for additional stromal factors that significantly influence cancer cell behavior and drug response. For example, incorporating immune cells or endothelial cells into the organoid culture could more accurately replicate the complexity of a real tumor under patient-specific conditions, thereby yielding more reliable results.

Conclusion

In summary, we developed a simplified 3D co-culture organoid model of TNBC incorporating epithelial cells and primary fibroblasts, allowing us to investigate the role of fibroblasts in modulating tumor behavior. Our results demonstrated that the presence of fibroblasts significantly increased doxorubicin resistance, promoted structural compactness, and enhanced directional migration within the organoid environment. These findings suggest that even in the absence of fibroblast transformation, fibroblasts can contribute to a protumorigenic niche through paracrine signaling and matrix remodeling. Importantly, the observed enhancement in drug resistance and migration occurred without the inclusion of immune or endothelial components, highlighting the intrinsic capacity of fibroblasts to alter tumor response and morphology. This underscores the necessity of incorporating stromal elements in organoid-based cancer models to more accurately reflect in vivo conditions and drug responses. Our model offers a minimal yet informative platform to explore epithelial–stromal interactions in TNBC. Future studies could expand this model by including immune cells, endothelial cells, or extracellular matrix substitutes to better recapitulate the tumor microenvironment and improve predictive accuracy for therapy testing.

Conflict of Interest

The authors declared that they have no conflict of interest.

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