

Hypoxic Breast Cancer Cells Modulate DRG Axon Outgrowth in a Tumor Aggressiveness-Dependent Manner

 Neşe AYŞİT^{1,2},  Esra ALTINTAŞ²

¹Department of Medical Biology, School of Medicine, İstanbul Medipol University, İstanbul, Türkiye

²Health Science and Technologies Research Institute (SABITA), İstanbul Medipol University, İstanbul, Türkiye

ABSTRACT

Introduction: Tumor-nerve interactions play a critical role in cancer progression, invasion, and metastasis. However, how tumor aggressiveness and hypoxic conditions influence neuronal remodeling remains incompletely understood in breast cancer.

Methods: This study investigated the effects of highly metastatic 4T1 and less aggressive EMT6 breast cancer cells, as well as their conditioned media, on dorsal root ganglion (DRG) axon elongation under normoxic and hypoxic conditions. Eight experimental groups were analyzed. Axon lengths were quantified using NeuronJ software in triplicate experiments. Statistical analyses were performed using one-way ANOVA followed by Tukey's post-hoc test.

Results: Under normoxic conditions, 4T1 cells induced the most pronounced axon elongation compared to EMT6 cells and control

groups. In contrast, hypoxia consistently suppressed neurite extension across all experimental conditions. Additionally, conditioned media alone exerted weaker effects than direct co-culture systems, indicating the importance of juxtacrine signaling and direct cell-to-cell interactions in tumor-neuron communication.

Conclusion: These findings demonstrate that tumor aggressiveness and oxygen availability differentially regulate neuronal remodeling in breast cancer. The results highlight neuron-tumor crosstalk as a potential therapeutic target in breast cancer progression.

Keywords: Axon elongation, breast cancer, dorsal root ganglia (DRG), hypoxia neuron-tumor interaction, tumor microenvironment

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INTRODUCTION

Emerging evidence increasingly recognizes the peripheral nervous system as an active and integral component of cancer biology, extending beyond its conventional role in sensory transmission to influence tumor initiation, progression, and therapeutic responsiveness (1). Cancer-nerve interactions have gained growing attention as critical determinants of disease behavior, particularly through mechanisms such as tumor-induced axonogenesis and perineural invasion (2). These processes reflect a dynamic bidirectional communication between neoplastic cells and neural structures, with significant implications for both tumor progression and neural plasticity (3).

Breast cancer provides a clinically relevant framework for exploring the neurobiological dimensions of tumor progression (4). Tumor-derived neurotrophic mediators and axon guidance molecules can promote neural sprouting toward the tumor microenvironment, while neural signaling, in turn, contributes to tumor proliferation, invasion, angiogenesis, and treatment resistance (5). This reciprocal relationship underscores the importance of neural remodeling as a fundamental yet underexplored aspect of tumor-host interactions.

Preclinical mammary tumor models enable mechanistic insights into how tumor aggressiveness influences neural responses (6). The highly

Highlights

- Highly metastatic breast cancer cells strongly promote DRG axon elongation
- Hypoxia suppresses neurite outgrowth regardless of tumor aggressiveness
- Direct tumor-neuron contact induces stronger neuronal remodeling than media
- Tumor aggressiveness differentially regulates neuron-tumor crosstalk

metastatic 4T1 cell line exhibits pronounced neurotrophic activity and closely models aggressive triple-negative breast cancer (7), whereas EMT6 cells represent a less aggressive and more hormonally responsive phenotype (8). Comparative investigation of these models provides a unique opportunity to elucidate subtype-specific effects on neuronal growth and plasticity (9).

Correspondence Address: Neşe Ayşit, Department of Medical Biology, School of Medicine, İstanbul Medipol University, İstanbul, Türkiye • E-mail: naysit@medipol.edu.tr

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Hypoxia, a hallmark feature of the tumor microenvironment, plays a pivotal role in shaping both tumor biology and neural responses (10). Through activation of hypoxia-inducible signaling pathways, hypoxic stress can modulate the expression of neurotrophic factors, angiogenic mediators, and axon guidance molecules, thereby influencing neuronal remodeling in a context-dependent manner (11). However, the impact of hypoxia on tumor-nerve interactions in breast cancer remains incompletely understood (12).

In the present study, dorsal root ganglion (DRG) neurite outgrowth was quantitatively evaluated using direct co-culture and conditioned media paradigms involving 4T1 and EMT6 breast cancer cells under normoxic and hypoxic conditions. This experimental framework enabled systematic investigation of how tumor aggressiveness and microenvironmental oxygen availability influence neuronal remodeling processes.

Based on accumulating evidence indicating that aggressive tumor phenotypes exhibit enhanced neurotrophic signaling and distinct responses to hypoxic stress, we hypothesized that hypoxic breast cancer cells differentially regulate DRG axon outgrowth in an aggressiveness-dependent manner. Specifically, we proposed that highly metastatic 4T1 cells exert greater modulatory effects on neurite extension compared with the less aggressive EMT6 phenotype, reflecting subtype-specific tumor-nerve interaction mechanisms mediated through both paracrine and contact-dependent pathways.

METHODS

Adult female Beta III-Tubulin-GFP transgenic mice (strain 4779; 6–8 weeks old; $n=10$) were obtained from the Experimental Animal Research Unit (MEDİTAM) Experimental Research Facilities of Istanbul Medipol University, and all procedures were conducted in accordance with institutional guidelines for animal care and use.

Following euthanasia under deep anesthesia, dorsal root ganglia (DRGs) were carefully dissected under sterile conditions using microsurgical techniques. Dorsal root ganglion tissues were incubated in 1% collagenase solution for 45 minutes, followed by three washes with Hanks' balanced salt solution (HBSS). Subsequently, tissues were exposed to 0.25% trypsin (10% working solution) in Neurobasal A (NBA) medium for 15 minutes and mechanically dissociated by gentle trituration using a pipette. After mechanical dissociation, 0.1% DNase was added to the cell suspension to prevent cellular aggregation. Neuronal purification was further achieved using Percoll gradient centrifugation, after which primary DRG neurons were plated onto culture dishes pre-coated with poly-L-lysine and laminin. Cultures were maintained in Neurobasal A medium supplemented with B27, L-glutamine, and penicillin/streptomycin, and incubated at 37°C in a humidified atmosphere containing 5% CO₂.

Murine breast cancer cell lines with distinct metastatic potential, including the highly metastatic 4T1 and the less aggressive EMT6 cell line, were cultured under standard conditions. For direct co-culture experiments, cancer cells were added to the same culture wells following neuronal adhesion, allowing direct cell–cell contact between tumor cells and DRG neurons.

To assess paracrine effects, conditioned media collected from 24-hour cancer cell cultures were centrifuged to remove cellular debris and applied to DRG neuronal cultures after neuronal attachment, without direct physical contact between tumor cells and neurons.

Experimental groups consisted of DRG neurons cultured alone (control), DRG co-cultured with 4T1 cells, DRG treated with 4T1 conditioned

medium, DRG co-cultured with EMT6 cells, and DRG treated with EMT6 conditioned medium. Each experimental condition was evaluated under both normoxic (21% O₂) and hypoxic conditions, with hypoxia induced by exposure to 1% O₂ for 6 hours using a controlled hypoxia chamber.

Neurite outgrowth was assessed by fluorescence microscopy based on GFP expression in β III-tubulin-positive neurons, with images acquired from 10 randomly selected microscopic fields per experimental group. Axon lengths were quantified using NeuronJ software, and all experiments were performed in three independent biological replicates. Statistical analyses were conducted using GraphPad Prism version 10.

Data were analyzed using one-way analysis of variance (ANOVA) followed by Tukey's post hoc test, and results were expressed as mean $\pm$ standard error of the mean (SEM), with $p < 0.05$ considered statistically significant.

ChatGPT was used solely for language editing and did not influence the scientific content of the manuscript.

RESULTS

Dorsal root ganglion neurite outgrowth was significantly influenced by tumor subtype, interaction modality, and microenvironmental oxygenation. Under normoxic conditions (21% O₂), exposure to EMT6-conditioned medium resulted in significantly greater axon elongation compared with both EMT6 co-culture and control conditions (Figure 1). In contrast, no significant difference was observed between the EMT6 co-culture and control groups. A similar pattern was identified in the aggressive 4T1 model, where conditioned medium significantly increased neurite extension relative to both co-culture and control conditions, while direct co-culture did not differ significantly from control (Figure 2). These findings indicate that tumor-derived soluble factors play a dominant role in promoting neurite outgrowth under normoxic conditions.

Under hypoxia (1% O₂), the interaction dynamics shifted. In the EMT6 model, both co-culture and conditioned medium significantly increased axon length compared with control, whereas no significant difference was detected between the two interaction modalities (Figure 3). Similarly, in the 4T1 model, both co-culture and conditioned medium significantly enhanced neurite elongation relative to control under hypoxic conditions, with comparable effects between interaction modalities (Figure 4). These observations suggest that hypoxia alters the relative contribution of contact-dependent and paracrine tumor–neuron signaling.

Direct comparison of tumor models under normoxic conditions revealed that conditioned media derived from both EMT6 and 4T1 significantly promoted neurite outgrowth compared with control and corresponding co-culture groups, while no significant differences were observed between tumor subtypes within the same interaction paradigm (Figure 5). However, under hypoxia, neurite extension was preferentially enhanced in the aggressive 4T1 model, whereas EMT6-associated conditions did not produce statistically significant increases relative to control (Figure 6), indicating that tumor aggressiveness modulates the neural response to hypoxic stress.

Within-model comparisons further demonstrated that hypoxia significantly reduced conditioned medium-induced neurite outgrowth in the EMT6 model, while direct co-culture-mediated axon elongation remained unchanged (Figure 7). In contrast, in the 4T1 model, conditioned medium-induced neurite extension differed significantly between normoxic and hypoxic conditions, whereas co-culture responses

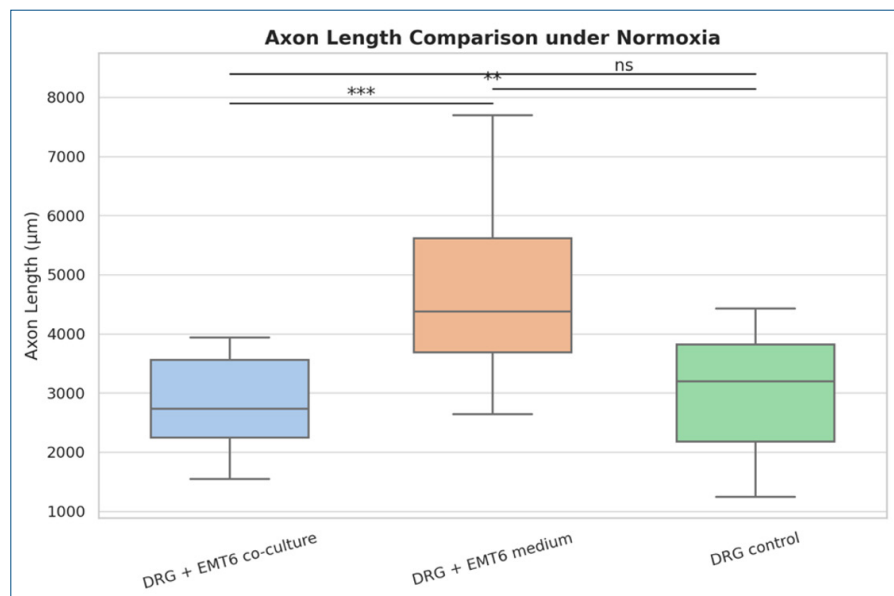


Figure 1. Comparison of DRG axon length among EMT6 co-culture, EMT6-conditioned medium, and control groups under normoxic conditions.

directly co-cultured with EMT6 breast cancer cells, or exposed to EMT6-derived conditioned medium under normoxia (21% O₂). Axon length was quantified and statistically compared using one-way ANOVA followed by Tukey's post hoc test. EMT6-conditioned medium significantly increased axon length compared with both the EMT6 co-culture and control groups, whereas no significant difference was observed between the EMT6 co-culture and control conditions. Data are presented as box-and-whisker plots showing the median (center line), interquartile range (box), and minimum-maximum values (whiskers). **p < 0.01; ***p < 0.001; ns, not significant.

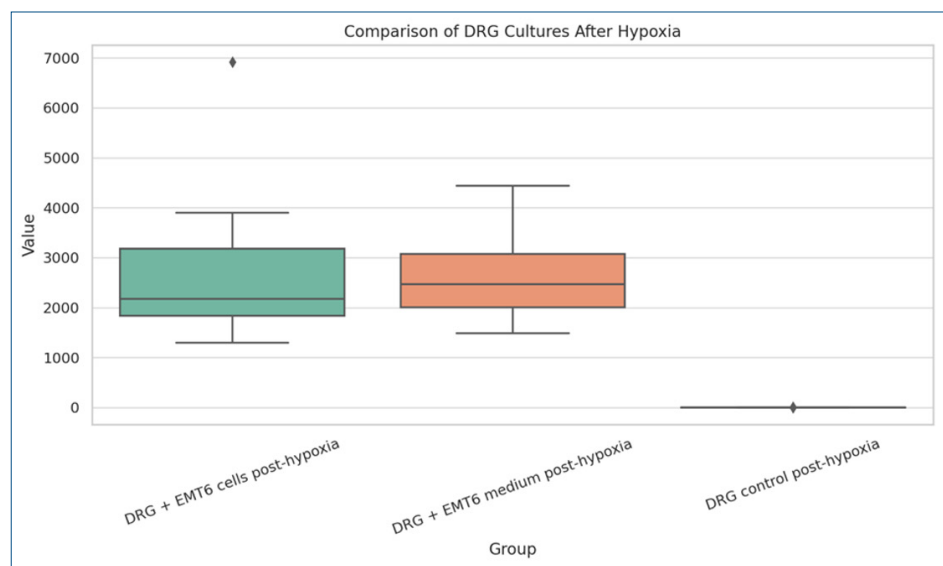


Figure 2. Comparison of DRG axon length among 4T1 co-culture, 4T1-conditioned medium, and control groups under normoxic conditions.

Primary DRG neurons were cultured alone (control), directly co-cultured with highly metastatic 4T1 breast cancer cells, or exposed to 4T1-derived conditioned medium under normoxia (21% O₂). Axon length was quantified and statistically compared using one-way ANOVA followed by Tukey's post hoc test. The 4T1-conditioned medium significantly increased axon length compared with both the 4T1 co-culture and control groups, whereas no significant difference was observed between the co-culture and control conditions. Data are presented as box-and-whisker plots showing the median (center line), interquartile range (box), and minimum-maximum values (whiskers). *p < 0.05; ns, not significant.

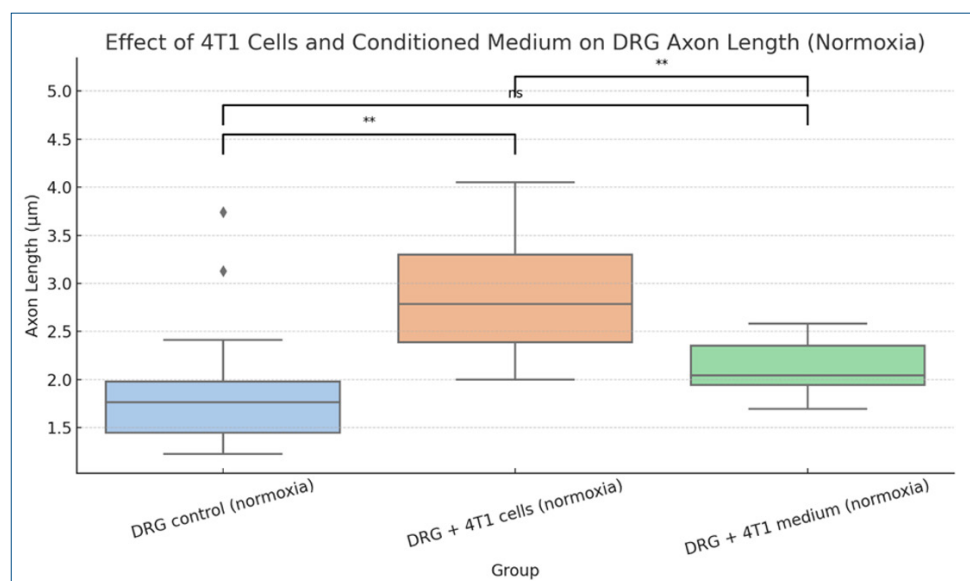


Figure 3. Comparison of DRG axon length among EMT6 co-culture, EMT6-conditioned medium, and control groups under hypoxic conditions.

Primary DRG neurons were cultured alone (control), directly co-cultured with EMT6 breast cancer cells, or exposed to EMT6-derived conditioned medium under hypoxia (1% O₂). Axon length was quantified and statistically compared using one-way ANOVA followed by Tukey's post hoc test. Both EMT6 co-culture and EMT6-conditioned medium significantly increased axon length compared with the control group, whereas no significant difference was observed between the co-culture and conditioned medium conditions. Data are presented as box-and-whisker plots showing the median (center line), interquartile range (box), and minimum-maximum values (whiskers). *p < 0.05; **p < 0.01; ns, not significant.

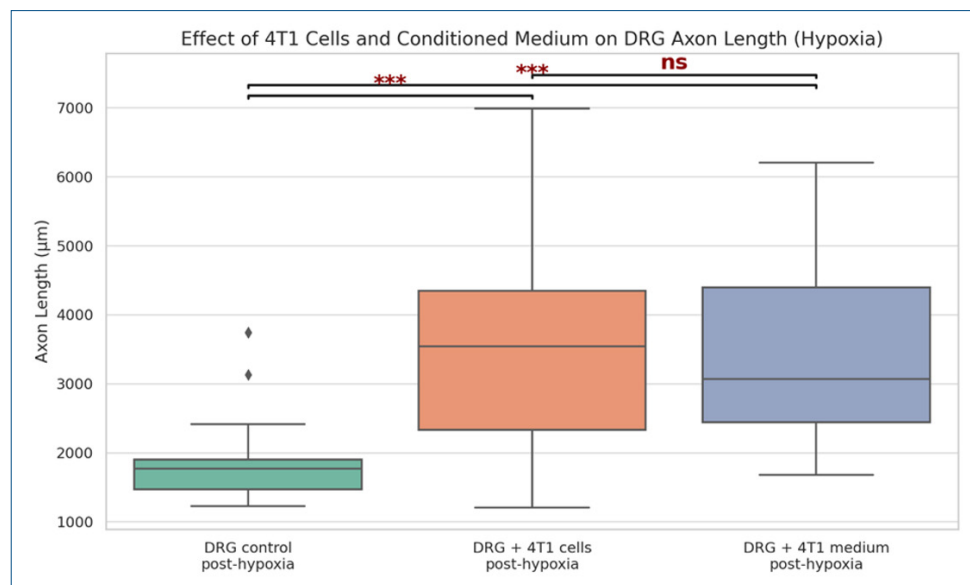


Figure 4. Comparison of DRG axon length among 4T1 co-culture, 4T1-conditioned medium, and control groups under hypoxic conditions.

Primary DRG neurons were cultured alone (control), directly co-cultured with highly metastatic 4T1 breast cancer cells, or exposed to 4T1-derived conditioned medium under hypoxia (1% O₂). Axon length was quantified and statistically compared using one-way ANOVA followed by Tukey's post hoc test. Both 4T1 co-culture and 4T1-conditioned medium significantly increased axon length compared with the control group, whereas no significant difference was observed between the co-culture and conditioned medium conditions. Data are presented as box-and-whisker plots showing the median (center line), interquartile range (box), and minimum-maximum values (whiskers). ***p < 0.001; ns, not significant.

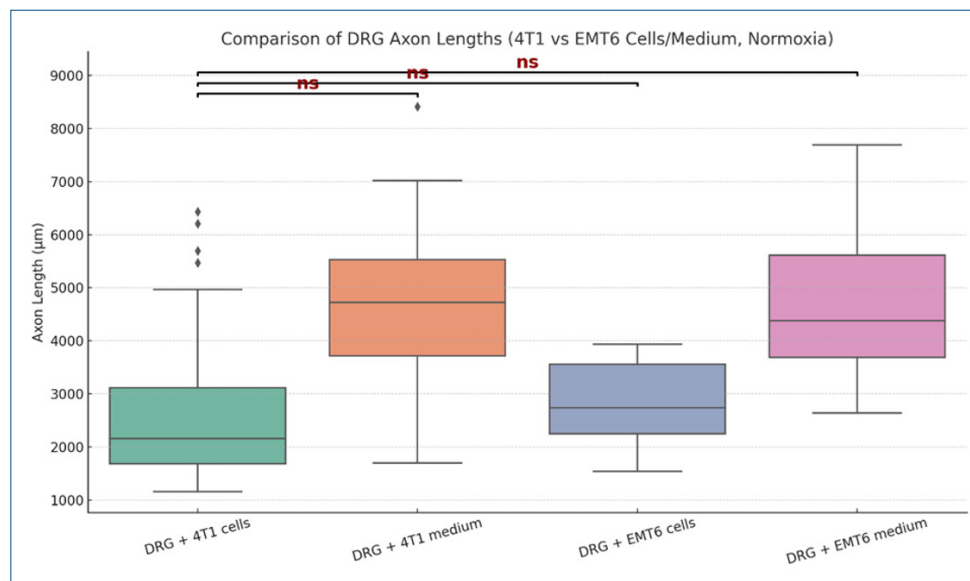


Figure 5. Comparative analysis of DRG axon length across EMT6 and 4T1 breast cancer models under normoxic conditions.

Primary DRG neurons were cultured alone (control), directly co-cultured with EMT6 or highly metastatic 4T1 breast cancer cells, or exposed to conditioned media derived from each cell line under normoxia (21% O₂). Axon length was quantified and statistically compared using one-way ANOVA followed by Tukey's post hoc test. Conditioned media from both EMT6 and 4T1 significantly increased axon length compared with the control and corresponding co-culture groups, whereas no significant differences were observed between EMT6- and 4T1 co-culture conditions or between EMT6- and 4T1-conditioned media. Data are presented as box-and-whisker plots showing the median (center line), interquartile range (box), and minimum-maximum values (whiskers). **p < 0.01; ***p < 0.001; ns, not significant.

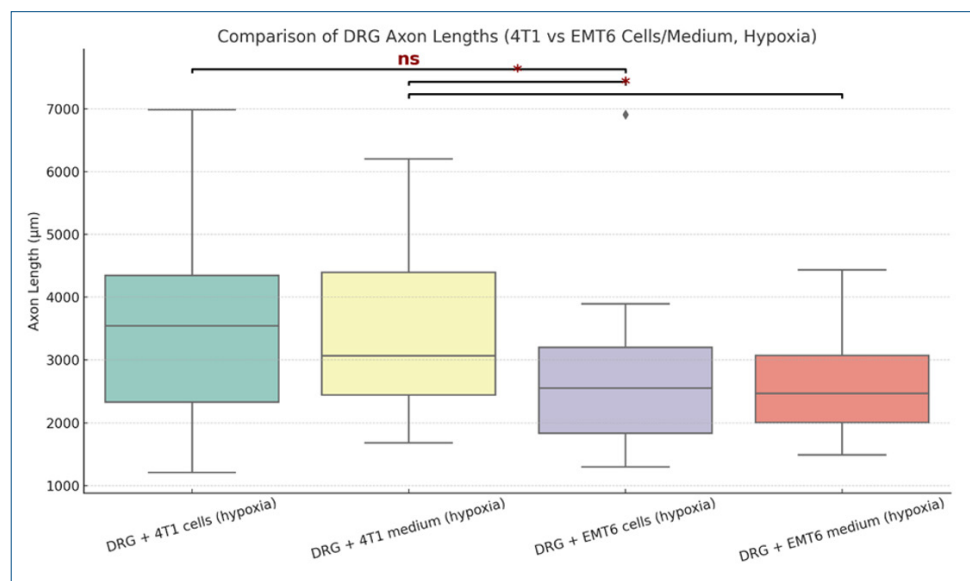


Figure 6. Comparative analysis of DRG axon length across EMT6 and 4T1 breast cancer models under hypoxic conditions.

Primary DRG neurons were cultured alone (control), directly co-cultured with EMT6 or highly metastatic 4T1 breast cancer cells, or exposed to conditioned media derived from each tumor cell line under hypoxia (1% O₂). Axon length was quantified and statistically compared using one-way ANOVA followed by Tukey's post hoc test. Neurite outgrowth was significantly increased in both the 4T1 co-culture and 4T1-conditioned medium groups compared with control, whereas EMT6-associated conditions did not produce statistically significant changes. Data are presented as box-and-whisker plots showing the median (center line), interquartile range (box), and minimum-maximum values (whiskers). **p < 0.01; ***p < 0.001; ns, not significant.

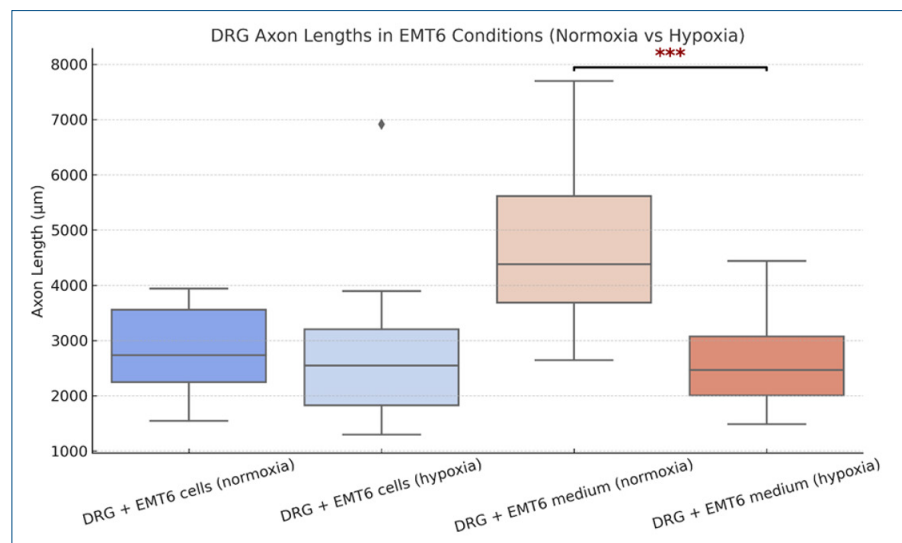


Figure 7. Effects of hypoxia on DRG axon length in EMT6 tumor–neuron interaction models.

Primary DRG neurons were either directly co-cultured with EMT6 breast cancer cells or exposed to EMT6-derived conditioned medium under normoxic (21% O₂) or hypoxic (1% O₂) conditions. Axon length measurements revealed that hypoxia significantly reduced conditioned medium-induced neurite outgrowth, whereas no significant difference was observed between normoxic and hypoxic co-culture conditions. Statistical comparisons between normoxic and hypoxic groups were performed using independent two-tailed t-tests. Data are presented as box-and-whisker plots showing the median (center line), interquartile range (box), and minimum–maximum values (whiskers). *p < 0.05; ***p < 0.001; ns, not significant.

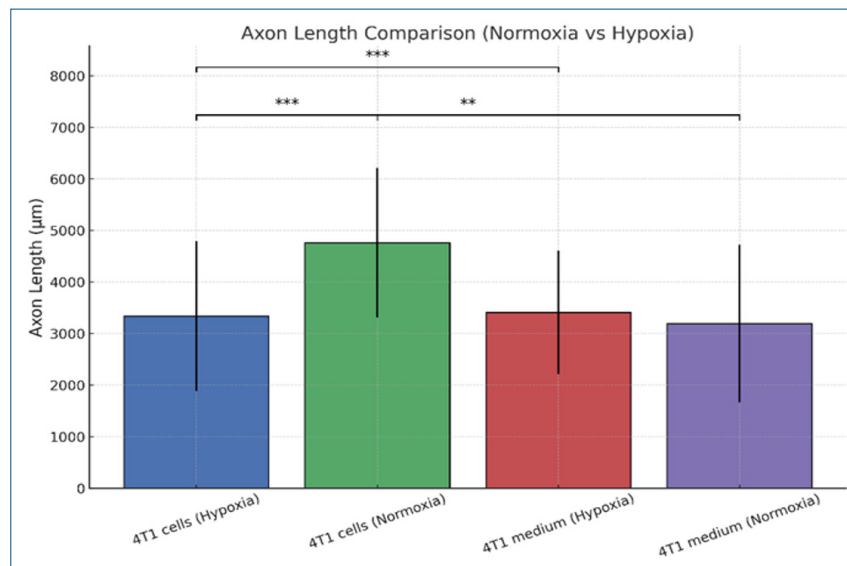


Figure 8. Effects of hypoxia on DRG axon length in 4T1 tumor–neuron interaction models.

Primary DRG neurons were either directly co-cultured with highly metastatic 4T1 breast cancer cells or exposed to 4T1-derived conditioned medium under normoxic (21% O₂) or hypoxic (1% O₂) conditions. Axon length measurements revealed no significant difference between normoxic and hypoxic co-culture conditions, whereas conditioned medium-induced neurite outgrowth differed significantly between oxygenation states. Statistical comparisons between normoxic and hypoxic groups were performed using independent two-tailed t-tests. Data are presented as box-and-whisker plots showing the median (center line), interquartile range (box), and minimum–maximum values (whiskers). *p < 0.01; ns, not significant.

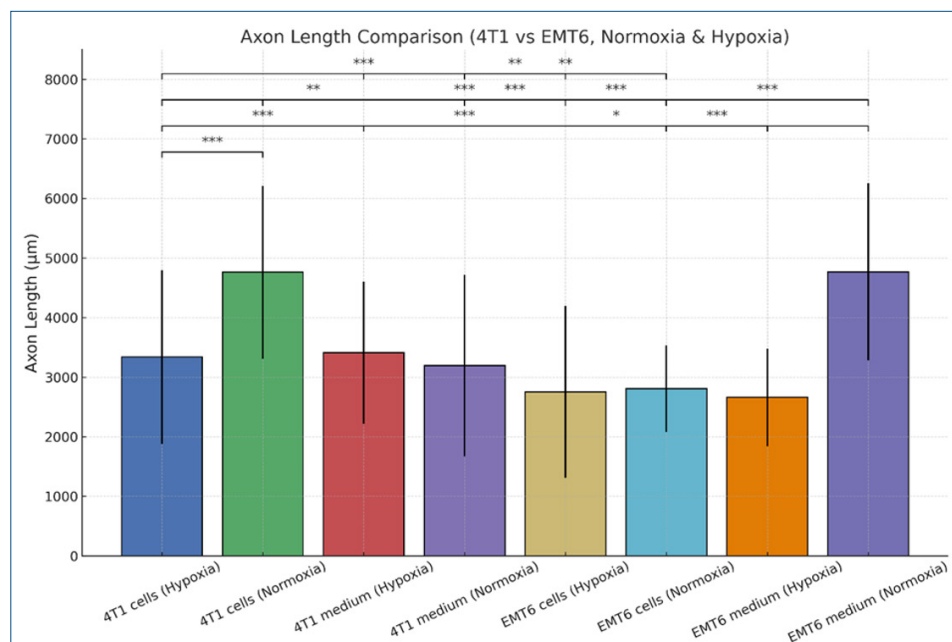


Figure 9. Differential effects of hypoxia on tumor-induced DRG axon outgrowth in EMT6 and 4T1 breast cancer models.

Primary dorsal root ganglion (DRG) neurons were either directly co-cultured with tumor cells or exposed to tumor-derived conditioned media under normoxic (21% O₂) or hypoxic (1% O₂) conditions. In the EMT6 model, hypoxia significantly reduced conditioned medium-induced axon outgrowth, whereas no significant difference was observed between normoxic and hypoxic co-culture conditions. In contrast, in the aggressive 4T1 model, hypoxia moderately altered conditioned medium-induced neurite outgrowth while preserving contact-dependent tumor–neuron signaling. Statistical comparisons between normoxic and hypoxic groups were performed using independent two-tailed t-tests. Data are presented as box-and-whisker plots showing the median (center line), interquartile range (box), and minimum–maximum values (whiskers). **p < 0.01; ***p < 0.001; ns, not significant.

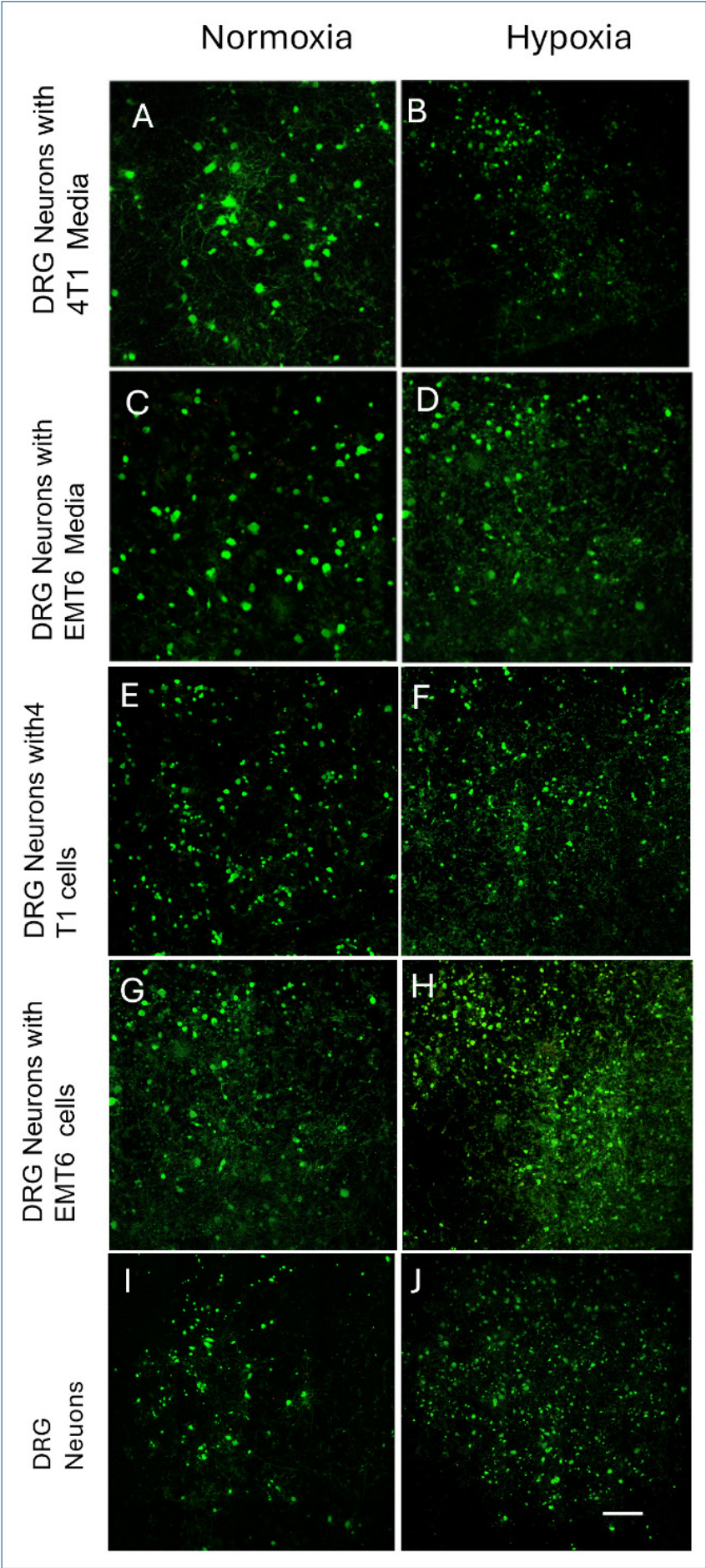


Figure 10. Representative confocal images of dorsal root ganglion (DRG) neurite outgrowth under all experimental conditions are presented in Figure 10.

Representative confocal images of dorsal root ganglion (DRG) neurite outgrowth under the indicated experimental conditions. DRG neurons were cultured with EMT6 or 4T1 breast cancer cells (direct co-culture) or exposed to their conditioned media under normoxic (21% O₂) or hypoxic (1% O₂, 6 h) conditions. Panels A-J represent the corresponding experimental conditions according to tumor subtype, culture condition, and oxygenation status. Scale bars = 20 µm. Direct cell-cell contact promoted more robust neurite extension than conditioned media, whereas hypoxia generally inhibited axon elongation across the experimental groups.

remained statistically comparable (Figure 8). These results indicate that hypoxia selectively modulates paracrine tumor-derived neurotrophic signaling pathways.

Integrated multi-panel analysis confirmed that hypoxia exerts differential effects on tumor-induced DRG axonogenesis depending on tumor aggressiveness (Figure 9). Specifically, hypoxia markedly suppressed conditioned medium-mediated neurite outgrowth in the EMT6 model without affecting contact-dependent signaling, whereas in the 4T1 model, hypoxia moderately altered paracrine effects while preserving direct tumor–neuron interaction-driven axon elongation.

Collectively, these findings demonstrate that tumor aggressiveness, interaction modality, and oxygenation status jointly determine the magnitude and pattern of DRG neurite outgrowth. Representative confocal images illustrating DRG neurite outgrowth across all experimental conditions are shown in Figure 10.

DISCUSSION

Our results demonstrate that tumor aggressiveness and oxygenation status cooperatively regulate tumor-associated neural remodeling, providing further evidence that the peripheral nervous system (PNS) is not merely a bystander but an active driver of cancer progression (13). The finding that 4T1 cells under normoxia promoted the most robust DRG neurite elongation aligns with studies showing that highly metastatic, triple-negative breast cancer cells secrete abundant nerve growth factor (NGF), brain-derived neurotrophic factor (BDNF), and glial cell line-derived neurotrophic factor (GDNF), which activate TrkA, TrkB, and RET receptors on sensory neurons to stimulate neurite sprouting (14). These neurotrophic cues, together with axon guidance molecules such as SEMA3C, netrin-1, and ephrin-A ligands, not only promote neuronal recruitment but also potentiate tumor motility and invasion through reciprocal neurotransmitter release (15).

By contrast, hypoxia (1% O₂, 6 h) uniformly suppressed neurite growth, consistent with prior reports that hypoxia activates HIF-1 α and HIF-2 α pathways, redirecting metabolic resources to glycolysis while downregulating neurotrophic factor secretion (16). Hypoxia can also induce repulsive axon guidance cues such as SEMA3A, VEGF-A, and ephrin-A5, which destabilize neuronal growth cones and limit neurite extension (17). These mechanisms have been observed in multiple cancers, including glioblastoma and pancreatic adenocarcinoma, where hypoxic zones exhibit diminished axonogenesis despite pro-angiogenic signaling (18).

Another key observation is that conditioned media alone induced weaker neurite outgrowth than direct co-culture, underscoring the critical role of cell-surface adhesion molecules such as NCAM1, L1CAM, and integrins in juxtacrine neuron–tumor signaling (19). These molecules facilitate localized paracrine gradients and synapse-like interactions that soluble factors alone cannot replicate (20).

The preferential axonogenesis induced by 4T1 over EMT6 cells highlights how tumor phenotype governs neural remodeling, echoing clinical evidence that perineural invasion and tumor innervation correlate with early recurrence and poor survival in breast, pancreatic, and prostate cancers (21). Sympathetic innervation, in particular, has been linked to enhanced metastatic spread via adrenergic signaling (22). Thus, the tumor–nerve axis may serve as a “metastatic highway”, amplifying cancer cell dissemination.

These findings suggest that targeting neurotrophic (NGF/Trk, BDNF/TrkB), axon guidance (SEMA3-neuropilin, netrin-DCC), and adhesion molecule

pathways could impair tumor-associated axonogenesis and potentially curb metastatic progression (23,24). Preclinical studies employing neuronal ablation, β -adrenergic blockade, and semaphorin-neuropilin inhibition have already demonstrated reductions in tumor growth and metastasis (25). Our results support further exploration of such strategies, particularly in triple-negative breast cancer, where conventional therapies remain limited.

This study establishes that breast cancer-induced axon regeneration is shaped by both tumor subtype and microenvironmental oxygenation.

- 4T1 cells under normoxia serve as potent inducers of DRG neurite elongation, reflecting their aggressive, neurotrophin-rich phenotype.
- Hypoxia consistently suppresses axonal growth, likely via HIF-driven metabolic reprogramming and induction of repulsive axon guidance molecules.
- Direct neuron–tumor contact, rather than soluble secreted factors alone, is indispensable for maximal neurite extension.
- Tumor aggressiveness directly correlates with neural remodeling, with 4T1 consistently surpassing EMT6 in pro-neurogenic effects.

These findings underscore the neurobiological dimension of breast cancer aggressiveness and highlight the tumor–nerve axis as a viable therapeutic target. Future studies integrating in vivo longitudinal imaging, neural ablation, and pharmacological inhibition of NGF/Trk and semaphorin-neuropilin pathways will be essential to determine whether disrupting this axis can limit metastasis and improve survival. Such approaches may also hold relevance for veterinary oncology, where similar neuron–tumor interactions have been implicated.

Limitations of This Study

Despite the strengths of the present study, we acknowledge that its scope is limited by the lack of a detailed mechanistic dissection of the molecular pathways driving tumor-induced axon regeneration. While our findings strongly implicate hypoxia-responsive signaling, neurotrophin availability, and contact-dependent neuron–tumor interactions, the precise signaling mechanisms underlying these effects were not directly interrogated. Elucidating these pathways will be essential to further enhance the biological and translational impact of this work and will be addressed in a broader, more comprehensive future project.

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Peer-review: Externally peer-reviewed.

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