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Establishment of a metastatic patient-derived xenograft model of breast carcinoma

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In this study, we investigated the development and application of patient-derived xenograft (PDX) models for metastatic breast carcinoma. Orthotopic PDX models were established from tumor tissue of invasive ductal breast carcinoma patients without prior neoadjuvant therapy. Following successful tumor engraftment in immunodeficient NOD SCID gamma (NSG) mice, primary tumors were surgically excised using a procedure simulating radical mastectomy, enabling extended post-operative survival for the development of eventual metastases. Tumor growth, the presence of lung metastases, along with their histopathological features and immunohistochemical profiles, were evaluated. Three stable and transplantable PDX models were successfully established. Spontaneous lung metastases developed in one model following surgical resection, and this metastatic capability was preserved across multiple *in vivo* passages. Despite the successful engraftment, significant discrepancies were observed between the immunohistochemical characteristics of the original patient tumors and their corresponding PDXs, particularly in hormone receptor status and Ki-67 proliferation marker expression. These findings highlight the challenges of maintaining molecular fidelity in traditional PDX models.

Key words: patient-derived xenografts; breast carcinoma; metastasis; immunohistochemical profiles; preclinical models

Breast cancer is the most common cancer in women worldwide. Based on the immunohistochemical expression of hormone receptors, including estrogen receptor (ER) and progesterone receptor (PR), and human epidermal growth factor receptor 2 (HER2), breast cancer is classified into four subtypes: luminal A, luminal B, HER2-positive, and triple-negative breast carcinoma (TNBC) [1]. Luminal A breast cancer is characterized by the presence of ER and/or PR, the absence of HER2, and low expression of the cell proliferation marker Ki-67 (<20%) [2]. Luminal B breast cancer exhibits a higher degree of malignancy and has a worse prognosis compared to luminal type A. It is also characterized by ER and/or PR, HER2 positivity, and high Ki-67 expression (>20%) [3]. The HER2-positive subtype accounts for 10–15% of breast carcinomas and is characterized by high HER2 expression without the expression of ER and PR [4]. Finally, TNBC, also referred to as basal-like breast cancer, exhibits ER-negative, PR-negative, and HER2-negative phenotype. TNBC is characterized by aggressiveness, early relapse, and a greater tendency to present at advanced stages [4]. Breast cancer frequently metastasizes to distant organs, most commonly the lungs, liver, bones, and brain. While the luminal breast cancer subtypes tend to metastasize to the bones, TNBC more often spreads to the lungs [5]. The development of distant metastases presents a major obstacle to effective cancer therapy and reduces the 5-year survival rate of patients with recurrent metastatic breast cancer to below 20% [6].

Even though preclinical *in vitro* cancer cell line models present a cost-effective and robust option for studying cancer cell biology, their lack of tumor heterogeneity and the absence of a complex tumor microenvironment limit

their translational application. Patient-derived xenograft (PDX) models were developed to address these limitations as they better reflect the tumor's complex nature, including the inter- and intra-tumor heterogeneity. PDX models are established by implanting human tumor tissue into highly immunodeficient mice and can be maintained through serial *in vivo* passages. These models are primarily used to evaluate the efficacy of chemotherapy or to characterize tumor cells and their microenvironment, as they closely resemble the patients' original tumors [7–9]. The commonly used models are highly immunodeficient NOD SCID gamma (NSG) mice. They share features with non-obese diabetic mice (NOD/ShiLtJ), including defects in innate immunity, a severe combined immunodeficiency (SCID) mutation, and an IL-2 receptor gamma chain deficiency. This results in a lack of mature T cells, B cells, and functional NK cells, as well as reduced cytokine signaling [10]. Tumors can be implanted heterotopically (most often subcutaneously) or orthotopically. For breast cancer PDX models, orthotopic implantation into mammary glands is preferred, as it more accurately recreates the tumor microenvironment and allows better monitoring of tumor growth. This facilitates interactions between tumor cells and stromal components, increasing the likelihood of metastasis and expanding the applicability of PDX models [11, 12]. To further replicate the characteristics of the original tumor, patient-derived cancer-associated fibroblasts (CAFs) or mesenchymal stromal cells (MSCs) can be co-transplanted with tumor cells into breast cancer PDX models [13, 14].

However, the growth rate of tumors from estrogen-dependent cancer cells is highly influenced by the mouse endogenous estrogen levels. Thus, estradiol pellets are often implanted into immunodeficient mice before luminal A/B tumor transplantation [9]. The implantation success rate for luminal breast carcinomas is very low: 9% for ER-positive primary tumors and 16% for metastatic ER-positive tumors. The success rate is higher for HER2-positive tumors, with 25% for primary tumors and 33% for metastatic tumors. The highest success rates are observed in TNBC, with 58% for primary tumors and 85% for metastatic tumors, partly due to their aggressive nature [15]. While the success rate of patient-to-mouse transplantation varies depending on the tumor type, the subsequent success rate of mouse-to-mouse transplantation is generally high. Therefore, if PDX is successfully established through the initial engraftment, it can be propagated into a statistically relevant cohort of mice by passaging tumors from the established PDX into a suitable number of animals [16, 17].

Metastatic spread presents a major cancer-related clinical challenge and a cause of death. Preclinical models of cancer metastasis are thus a valuable tool to study the processes related to cancer dissemination. Methods to generate *in vivo* metastatic breast cancer models usually involve orthotopic implantation or tail vein injection [18]. However, the orthotopic implantation requires a longer time period for the development of distant metastasis. Nevertheless, PDX cancer studies are constrained by bioethical regulations. According to the European Union Directive 2010/63/EU on the protection of animals used for scientific purposes, the maximum allowable tumor volume in murine models is not strictly defined but is generally accepted to be around 1,500 mm^3 for subcutaneous tumors. This poses a challenge for studying metastatic progression, particularly in the context of rapidly growing tumors. Therefore, the primary aim of our study was to develop PDX models suitable for investigating spontaneous lung metastasis formation by surgically excising the primary tumors and allowing the mice to survive, thereby enabling the development of eventual metastases.

Patients and methods

Patient samples. Fresh primary tumor tissues were obtained from patients with breast carcinoma without prior neoadjuvant therapy. All patients provided written informed consent for participation in the study and the use of their biological samples for research purposes (38-2022/EK OÚSA, TRUSK-003 NOU).

Patient T48 was diagnosed with mucinous luminal B invasive ductal carcinoma, grade 2, pT2 pN2b pMx, ER+ 95%, PR–, HER2–, Ki-67 5%. Patient T70 was diagnosed with luminal B intraductal breast carcinoma, grade 2, pT1c pN0 pMx, ER+ 90%, PR+ 95%, HER2–, Ki-67 20%. Patient T82 was diagnosed with luminal B intraductal breast carcinoma, grade 3, pT1c pN0 pMx, ER+ 99%, PR+ 1%, HER2+, Ki-67 90% and with confirmed germline mutations in the *BRCA1* and *CHEK2* genes. Patient T83 was diagnosed with intraductal breast carcinoma, grade 3, pT2 pN0 pM0, ER 2%, PR–, HER2–, Ki-67 50% (Summarized in Figure 1A).

PDX establishment. Female NSG mice were used for the establishment of the stable PDXs. The mice were housed in the authorized animal facility (SK UCH 02022). The study was approved by the Institutional Ethical Committee and the State Veterinary and Food Administration of the Slovak Republic (Registration Number Ro:1976/17-221). All animal experiments were conducted in compliance with the European Union Directive 2010/63/EU and institutional guidelines and approved protocols to ensure animal welfare. Ethical concerns inherent to PDX models were mitigated by reducing animal numbers through careful study design, closely monitoring tumor growth, and implementing humane endpoints to minimize suffering. Humane endpoints were defined as the time when the size of the secondary tumor reached approximately 1,000 mm^3 or signs of distress like lethargy, impaired mobility, and reduced food and water intake were observed.

Prior to surgery, 5 mg/kg of Meloxydil® (Ceva Santé Animale, France) was administered orally to reduce postoperative pain. Mice were anesthetized using inhalation anesthesia with isoflurane (Isoflutek® 1,000 mg/g, Alivira, Spain). Tumor pieces of T70, T82, and T83 patient samples

A

Sample	Sample type	Carcinoma type	Grade	Pathological stage	Germline mutations
T48	Organoids	IDC, Luminal B/ Invasive mucinous	Grade 2	pT2 pN2b pMx	
T70	Tissue	IDC, Luminal B	Grade 2	pT1c pN0 pMx	
T82	Tissue	IDC, Luminal B	Grade 3	pT1c pN0 pMx	*BRCA1, CHEK2*
T83	Tissue	IDC	Grade 3	pT2 pN0 pM0	

B

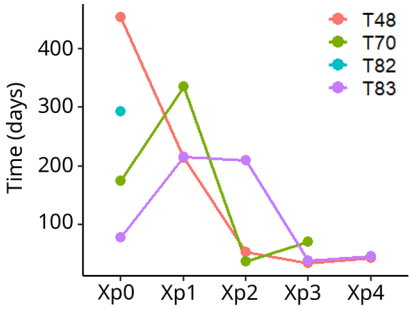

Figure 1. Sample characterization and establishment of patient-derived xenografts (PDX). A) Characterization of tumor samples, including patient clinical characteristics. B) Time intervals for PDX growth in each PDX generation. T82 sample generated PDX only in Xp0 and did not grow upon subsequent xenotransplantation. Abbreviation: IDC – invasive ductal carcinoma

were covered with ECM Gel from Engelbreth-Holm-Swarm murine sarcoma (Sigma-Aldrich, USA) and orthotopically bilaterally implanted into the 4th mammary fat pad of female NSG mice. A PDX model from the T48 patient sample was established by orthotopically injecting T48-derived organoids (in 5th passage, cultured according to Dekkers et al. [19]) resuspended in 50 µl of BME (Cultrex Reduced Growth Factor Basement Membrane Extract Type 2 Select, R&D Systems, USA) into a female NSG mouse. A 17β-estradiol pellet (0.18 mg with 60-day release, Innovative Research of America, USA) was implanted into the fat tissue in the interscapular region immediately prior to tumor implantation to support the engraftment of ER+ tumors.

Tumor excision and PDX passaging. Tumor xenografts were excised *en bloc* after reaching a volume of approximately 1,000 mm^3. The surgical wounds were sutured in anatomical layers after hemostasis. Mice were returned to their cage, awakened, and began moving within minutes after the surgery. Small fragments (2 mm × 2 mm) of the excised tumors were orthotopically transplanted into new recipient mice in the same manner as primary tumor tissue to establish subsequent PDX generations. Mice were euthanized either when secondary tumors reached the endpoint volume of approximately 1,000 mm^3 or when signs of serious distress and deteriorating health were observed.

Histological and immunohistochemical (IHC) staining. Formalin-fixed, paraffin-embedded tumor tissues were cut into 4 µm sections. Sections were stained with hematoxylin and eosin (H&E). For IHC analysis, deparaffinization, rehydration, and epitope retrieval (Target Retrieval solution high-pH, Dako, USA) were performed using a PT Link (Pre-Treatment module for tissue Specimens, Dako, USA) at 96 °C for 20 min. The slides were then washed with FLEX wash buffer (Dako, USA) prior to loading onto the automated Dako Autostainer_Link 48. Endogenous peroxidase activity was blocked by a 5 min incubation with FLEX peroxidase Block (Dako, USA). Sections were incubated with anti-human primary antibodies: Ki-67 (clone MIB-1,) Estrogen Receptor α (ER α, clone EP1), Progesterone Receptor (clone PgR 636) for 20 min at room temperature (FLEX, Dako, USA), GATA3 (L50-823, Cell Marque, USA) for 30 min at room temperature, followed by incubation with DakoCytomation EnVisio+System-HRP for 15 min. Positive staining was visualized by 3,3'-diaminobenzidine (DAB substrate-chromogen solution, Dako, USA) for 5 min (brown color). Counterstaining was performed with hematoxylin (FLEX, Dako, USA) for 10 min. Slides were washed between each incubation with FLEX wash buffer. Finally, slides were mounted using Faramount Mounting Medium, Aqueous (Dako, USA). The samples were evaluated in a light microscope Mateo FL (Leica, Germany). Immunohistochemical staining of HER2 was performed with HercepTest for Automated Link Platforms (Dako, USA), following a similar procedure as described above, except for the tissue dehydration process. Tissue slides were deparaffinized and rehydrated in a series of xylene and graded alcohols, followed by epitope retrieval using PT Link at 97 °C for 40 min.

Results

We successfully established three stable PDX models out of four transplanted tumor tissues of intraductal breast carcinoma: T48, T70, and T83. The samples represent two ER+ luminal B breast cancer subtypes and one ER-low breast carcinoma (Figure 1A). Established PDX models were successfully propagated *in vivo* over a series of xenopassages (Xp). The time necessary for the PDX growth was reduced in the latter PDX generations (Figure 1B). Upon euthanasia of the final PDX generation, small tumor fragments were cryopreserved for potential future implantation into immunodeficient mice to generate subsequent *in vivo* passages. Although the T82 patient tumor tissue representing the luminal B breast cancer subtype was successfully engrafted in the T82Xp0 mouse, the Xp1 generation could not be established.

IHC analysis (Figure 2, Table 1) revealed a loss of ER and PR expression across all derived PDX, despite three of the four corresponding patient tumors showing strong ER/PR positivity (90–99%) and one exhibiting low ER positivity (2%). Similarly, a loss of Ki-67 expression was observed in PDX models derived from T70 and T82. In contrast, low HER2 expression was detected in T70-derived PDX Xp2 and Xp3 mice, although the original patient tumor was HER2-negative.

The tumor excision procedure was effectively implemented to simulate an *en bloc*/radical mastectomy, mirroring surgical practices in clinical oncology and enabling the derivation of a xenograft model with spontaneous lung metastasis. The method was applied to all established xenograft lines. Of the four PDX lines, metastatic dissemination to the lungs was observed in all xenopassages of T48Xp mice (Figure 3A), with numerous bubble-like structures visually detected throughout the lung tissue, indicating extensive metastatic spread (Figure 3B).

To confirm the presence of breast cancer metastases in the lungs of T48 PDX models, we performed IHC staining for GATA3 (GATA binding protein 3), a marker used to identify breast cancer origin and typically highly expressed in luminal A and B subtypes. All analyzed samples showed strong GATA3 positivity (100%), confirming successful establishment of the metastatic xenograft model (Figures 3C, 3D).

Discussion

The primary objective of our study was to establish a PDX model for investigating breast cancer metastases. We successfully implemented a surgical *en bloc* tumor excision technique that enables postoperative survival of the mice, thereby providing sufficient time for the development of potential lung metastases. This approach allowed us to monitor metastatic progression over multiple PDX generations.

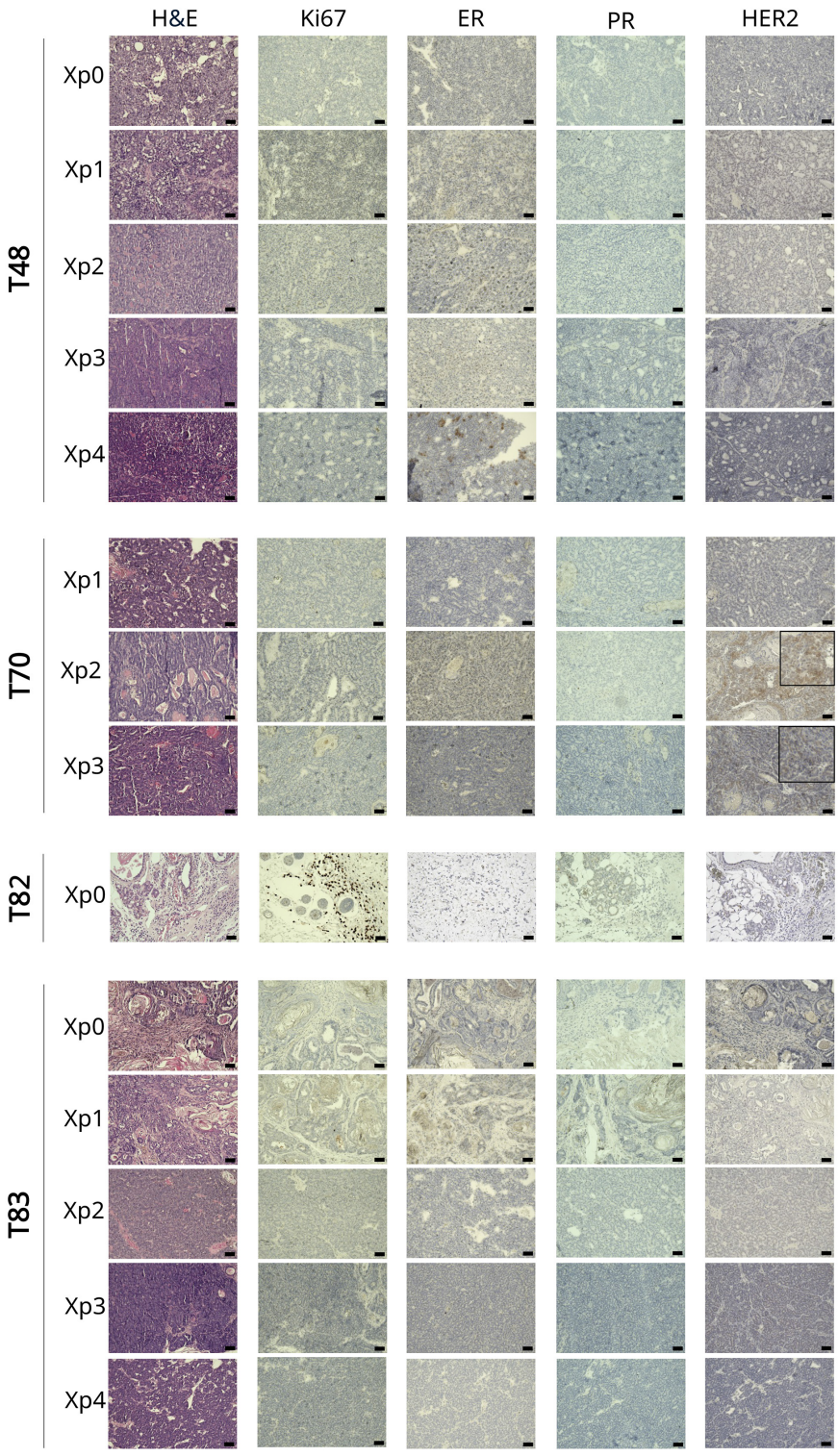

Figure 2. The representative immunohistochemical images of key markers in PDX captured at 200× (50 μm scale bar). Abbreviations: H&E – hematoxylin and eosin staining; ER – estrogen receptor; PR – progesterone receptor; HER2 – human epidermal growth factor receptor 2. Patient and PDX tumor tissue.

Interestingly, none of the T83-derived xenograft mice developed lung metastases across all PDX generations. The T83 patient was diagnosed with invasive ductal carcinoma (IDC), characterized by low ER expression (2%), PR negativity, and HER2 negativity. ER-low tumors share pathological and molecular features with TNBC, which is commonly associated with lung metastasis. For example, in Sweden, ER-low HER2-negative tumors are clinically managed as TNBC, using a ≥10% threshold for ER positivity [20]. This raises questions about the classification of ER-low tumors as a distinct subtype. Of the four PDX models (T48, T70, T82, and T83-derived), only the T48-derived model gave rise to lung metastases. This patient was diagnosed with mucinous luminal B IDC. Mucinous breast cancer accounts for only about 2% of breast cancer cases and is associated with higher age and a favorable prognosis [21, 22]. Luminal A/B subtypes are more likely to metastasize to bones than to lungs. This contrasts with the presence of extensive, bubble-like metastatic lesions observed in the lungs of T48-derived PDX mice in every generation. However, the development of spontaneous breast cancer bone metastases in orthotopic PDXs is rather uncommon [23]. Notably, the T48-derived xenograft was established from cultured patient-derived organoids rather than from fresh patient tumor tissue, as was the case for the other PDXs. Organoid culture may select for more stem-like or invasive cells, which are better adapted to grow *in vitro* and potentially more competent to metastasize *in vivo*. In contrast, fresh tumor implants contain a mix of cells, including many that are terminally differentiated or

Table 1. Summary of the immunohistochemical analysis of tumor tissues from patients and PDX.

		Ki-67	ER	PR	HER2
T48	Patient	5%	95%	–	–
	Xp0	–	–	–	–
	Xp1	–	–	–	–
	Xp2	–	–	–	–
	Xp3	–	–	–	–
	Xp4	–	–	–	–
T70	Patient	20%	90%	95%	–
	Xp1	–	–	–	–
	Xp2	–	–	–	+
	Xp3	–	–	–	+
T82	Patient	90%	99%	1%	+
	Xp0	60%	–	–	–
T83	Patient	50%	2%	–	–
	Xp0	–	–	–	–
	Xp1	–	–	–	–
	Xp2	–	–	–	–
	Xp3	–	–	–	–
	Xp4	–	–	–	–

Abbreviations: Ki-67 – marker of cellular proliferation; ER – estrogen receptor, PR – progesterone receptor, HER2 – human epidermal growth factor receptor 2. Notes: Percentages represent the proportion of cancer cells positive for marker expression; "–" indicates negative marker expression; "+" indicates positive marker expression

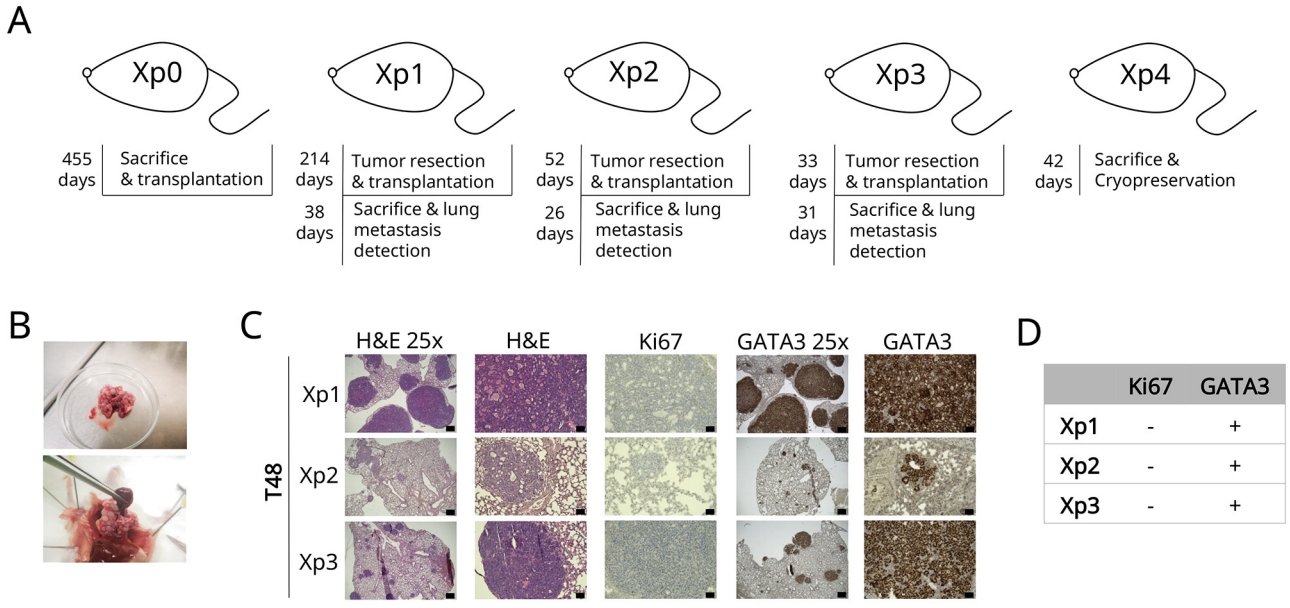

Figure 3. Derivation of metastatic PDX model. A) Experimental design for the derivation of the metastatic T48 PDX model. B) Representative images of lung metastases observed in the T48xp1 PDX model. C) The representative immunohistochemical images of H&E and GATA3 captured at 25× (400 µm scale bar) and H&E, Ki-67, and GATA3 captured at 200× (50 µm scale bar) from the lung tissue of the T48 PDX model. D) Summary of the immunohistochemical analysis of lung tissues from T48 PDX mice in three xenopassages. Abbreviations: H&E – hematoxylin and eosin staining; GATA3 – GATA binding protein 3. Notes: "–" indicates negative marker expression, "+" indicates positive marker expression

dependent on the original tumor microenvironment. Also, organoid systems impose a selective bottleneck, which means that only cells that survive and grow under 3D culture conditions persist. This could be beneficial for cells with higher metastatic potential, including epithelial-to-mesenchymal transition traits or survival under stress.

As metastases account for over 95% of breast cancer-related mortality, the establishment of a PDX model that reliably develops spontaneous lung metastases represents a valuable tool for *in vivo* studies on metastatic dissemination [24].

However, our study underscores both the potential and the limitations in replicating the complexity of breast carcinomas. In particular, the loss of ER/PR positivity and the loss of Ki-67 expression in the PDXs derived from ER/PR and Ki-67-positive tumors, as well as the emergence of low HER2 expression in T70Xp2 and Xp3 mice derived from a HER2-negative T70 tumor, highlight the challenges in preserving the IHC profile of the original tumor. Such alterations are well-documented in the literature, where tumor adaptation to the murine environment can lead to receptor loss or changes in marker expression [25]. The observed loss of ER/PR positivity could also be driven by the long time necessary for establishment of the primary PDX, exceeding the stated 60-day release of the implanted 17β-estradiol pellet. This could lead to tumor adaptation and selection of estrogen-independent cell clones. Additional estradiol supplementation or specific cancer cell injection methods could be considered to maintain hormonal receptor expression [26]. Our findings align with previous studies demonstrating that serial passaging and the lack of a complete human tumor microenvironment contribute to IHC marker variability. For example, we observed a complete loss of Ki-67 expression in T83Xp0, in contrast with the high proliferation marker expression (Ki-67 50%) in the patient's original tumor. Similarly, we observed the decrease of Ki-67 expression in T82Xp0 (60%) compared to the patient's tumor with high proliferation activity (Ki-67 90%). Studies have shown that the stromal and immune components of the tumor microenvironment, absent in standard PDX models, play a critical role in maintaining IHC marker fidelity [27]. Furthermore, the replacement of human stroma by murine-derived extracellular matrix and CAFs may alter signaling cues that regulate receptor expression and proliferation. Clonal bottlenecks during engraftment and serial passaging further amplify these effects, as certain subclones are better adapted to survive the murine tumor microenvironment. Adaptive metabolic and angiogenic responses to the murine host environment may also reshape tumor behavior, leading to the observed shifts in IHC profiles. Even the sampling bias caused by spatial heterogeneity in the original tumor may also contribute to the lower fidelity of PDX models [13, 28].

Addressing these limitations necessitates continued innovation in PDX model development. Humanized PDX models, which incorporate patient-derived CAFs or MSCs, have shown promise in enhancing the fidelity of tumor microenvironments. These co-transplantation strategies aim to restore key interactions between tumor cells and their surrounding stromal and immune components, potentially improving marker stability and therapeutic relevance [29, 30]. Another promising approach is the development of immunocompetent PDX models. By reintroducing key elements of the human immune system, researchers can better study immune-tumor interactions, which are critical for immunotherapy applications. For example, such models have successfully replicated immune responses in HER2-positive and TNBC subtypes, facilitating the evaluation of checkpoint inhibitors and other immunomodulatory therapies [31].

Our study demonstrates the development of a murine PDX model that spontaneously develops lung metastases. The majority of existing studies on breast cancer metastasis rely on the use of TNBC cell lines, such as MDA-MB-231, which are either orthotopically or intravenously injected into immunocompromised mice. These models are widely used because they consistently form lung metastases in a time-efficient and reproducible manner. However, while they are experimentally convenient, they lack the full complexity and heterogeneity of patient tumors. Other metastatic breast cancer models include syngeneic mouse models based on the utilization of the murine 4T1 cell line, mimicking stage IV TNBC and developing metastases in lungs, lymph nodes, liver, and bones [32], or genetically engineered mouse models, such as MMTV (mouse mammary tumor virus) strains. These MMTV mouse strains form lung metastases with variable incidence, ranging from about 10% to 100% [33]. However, spontaneous lung metastatic models derived from patient tumors better recapitulate the metastatic cascade, thus providing a more physiologically relevant platform for studying metastasis [34].

Despite these advantages, our findings also highlight key limitations of PDX models, particularly in preserving the molecular and cellular characteristics of the original tumor. Variations in marker expression and receptor status during serial passages emphasize the challenges of faithfully mimicking the patient tumor microenvironment. Therefore, integrating PDX models with complementary systems, such as patient-derived organoids or *ex vivo* tumor slice cultures, may offer a more comprehensive approach to understanding tumor evolution, heterogeneity, and treatment response in metastatic breast cancer.

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