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REVIEW ARTICLE

An Overview of the Bench to Bedside Models of Breast Cancer in the Era of Cancer Immunotherapy

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ABSTRACT

One of the barriers to novel treatment developments within breast cancer is the ability to prove efficacy in the preclinical setting before moving on to clinical trials. Preclinical models range from single cell monolayers to more sophisticated humanised PDXs systems each with their set of advantages and limitations. Modelling the immune environment in cold tumours, such as breast cancer can also be challenging as are currently no clearly defined markers that can stratify patients based on treatment response. Immune checkpoints receptors such as PD-L1 may not show predictive outcomes in this tumour type. Furthermore, the heterogeneity of breast cancer may be difficult to recapitulate at the bench side. In this review, we provide an overview of the available in vitro, in vivo and ex vivo models of breast cancer with consideration of how these may extrapolated to the investigation of the role of the immune system and immunotherapy developments in breast cancer.

Introduction

Breast cancer is the most common cancer in women. It is incredibly heterogeneous and has been classified into several subtypes based on cell receptor status which allow for stratification of treatments and prediction of prognosis. The invasiveness and metastatic potential of tumours is largely dependent on their subtype and treatment designated accordingly. The outcomes on early breast cancer have improved significantly over the last 25 years due to optimization of chemotherapy regimens and the addition of targeted therapies such as hormonal treatments and antibodies against the HER2 receptor. However, in the metastatic setting, although there is a trend to improved outcomes [1–3] particularly in the HER2+ patients, long-term prognosis remains poor and there is a clear need for more effaceable treatment options for these patients.

Development of novel therapies is dependent on the ability to prove efficacy in the preclinical setting. However, a large proportion of promising therapies have never made it to clinical trial. Part of this failure may be due to the limitations of preclinical models to mimic the complexity of the heterogeneous tumour microenvironment. The number of breast cancer models are vast, and the choice of model is often based on the question posed. Immortalized cell lines, for example, can be used to correctly identify new treatments, such as tamoxifen in ER+ breast cancer [4]. In humans, 80% of triple negative breast cancers (TNBC) are basal by molecular profiling [5,6] and the majority of the cell lines described have a basal molecular class which means treatments can be directed to the most common groups. However, some treatments may not produce effects within a 2D model, necessitating the use of more complex 3D modelling systems [7]. In addition, data published by Hollern et al [8] described that despite the vast heterogeneity within mouse models of breast cancer and these may or may not represent what is seen clinically. Furthermore, exploring the immune system within conventional models may pose more of a challenge as the immortalized cell lines used are often derived from a human source and are therefore only grown in immunosuppressed animal hosts.

The intention of this review is to provide an overview of the current available models of breast cancer (Table I) and the advantages, limitations, and challenges that each face when applied to novel and immunotherapeutic drug discoveries and how this may be extrapolated into future clinical trials. In this, readers will develop an understanding of the current in vitro, in vivo and ex vivo techniques.

Immunotherapy in breast cancer

Historically the mainstay of breast cancer treatment has been a strong chemotherapy backbone with clinical responses elicited from many chemotherapy agents including anthracyclines, taxanes, carboplatin and fluorouracil. Understanding of the cancer heterogenicity and selective targeting of aberrant pathways have increased the treatment repertoire. Currently treatment can be classified into chemotherapies, targeted therapies (including hormonal targets) and immunotherapies and are often administered in combination regimes to increase efficacy and decrease the occurrence of cancer resistance. Immunotherapies are a relatively new addition to the breast oncologist's tool kit and, if it's use mirrors other tumour types, it is likely to feature more prominently in coming years.

Modulation of the immune system using immune checkpoint inhibitors has transformed the treatment of solid malignancies such as melanoma, renal and lung cancer [20]. It's use in breast cancer was initially contentious as some argue that the lack of an inflamed immunogenically hot tumour microenvironment, particularly within the ER+HER2- group, will preclude a response to such treatments. As such, clinical studies of immunotherapies have been primarily limited to TNBC with proven clinical trial success.

Immunotherapy agents which have reached clinical trial stage can be classified into 3 areas; immune check point inhibitors, adoptive cell transfer and oncolytic viruses. Checkpoint inhibition has been the most well researched with 3 main agents described in breast cancer; pembrolizumab, atezolizumab and avelumab. Pembrolizumab is a humanized monoclonal IgG4 kappa antibody to the PD-1 receptor and blocks the interaction with PD-L1 and PD-L2 on tumour cells. It now sits as one of the standard treatment options for both early and metastatic breast cancer based on the results of 2 significant KEYNOTE trials. In the KEYNOTE 355 trial [21], 847 patients with advanced untreated TNBC were randomized to receive either chemotherapy plus placebo or chemotherapy plus pembrolizumab. In final interim analysis after 44.1 months of follow up, patients stratified by PD-L1 a high combined PD-L1 score of over 10 had a significant increase in overall survival from 23 to 16.1 months (P=0.0185). Additionally, the use of neoadjuvant pembrolizumab in early breast cancer has become a new standard of care in the UK following the KEYNOTE 522 trial, which revealed a decreased in cancer related event free survival in patients treated with combination neoadjuvant chemotherapy and pembrolizumab compared to chemotherapy verses placebo[22]

Table 1: Current models of cancer

Origin of cells	Model type	Advantages	Disadvantages	Examples of use with immunotherapies
Immortalized cell lines	2D monolayer	-Easy and cheap - Can look for direct cell to cell changes	-Unable to replicate TME -Cell lines may not be truly presentative of patient's cancer	Detection of CTLA-4 receptors on a number of immortalized cell lines allowed testing of a CTLA-4 inhibitor on these cell lines with a promising response [9]
	3D Spheroid	-Easy and cheap -Necrotic centre -Direct cell to cell interactions	-Unable to replicate TME -May not be truly presentative of patient's cancer -Lacks heterogenicity	Spheroids of HGC27 were incubated with T cells in a model to evaluate the cytotoxicity of the PD-1 blockade. These provided useful information about T cell cytotoxicity within this system [10].
	3D scaffold	- Some replication of TME -Can assess diffusion	-No vasculature structures -Cell lines may not be truly presentative of patient's cancer	Using a hydrogel scaffold, mutant Ad5-3Δ-A20T infected pancreatic stellate cells indicating improved viral spread within the microenvironment in this 3D hydrogel model that would have not been discernable in 2D culture [11].
	3D microfluidics	Able to assess perfusion or flow of substances using micro vessels	-Cell lines may not be truly presentative of patient's cancer -Lacks heterogenicity	A multicellular tumor-on-a-chip platform involving breast cancer cell lines (MCF7, MDA-MB- 231), monocytes and endothelial cells within a gelatin hydrogel was infused with T cells to assess T cell movement and cytokine release [12].
	Immunodeficient mouse model	- Allows more complex modelling of substances - Can use human derived cell lines	- Lacks immune system - Cancers may not be truly presentative of patient's cancer	Female NOD/SCID mice were used to general a model of pancreatic cancer which demonstrated immune cells enhanced the activity of gemcitabine, erlotinib and NK cells [13].
	Immunocompetent mouse model	-Allows more complex modelling of substances - Immune system present	-May not be truly presentative of patient's cancer -Differences in mouse and human immune response	C57BL/6 female mice were inoculated with a B16 melanoma cell line and treated with a CTLA-4 blockers and GMCSF vaccines which demonstrated efficacy and toxicity with autoimmune depigmentation [14]
	Humanized mouse model	- complex modelling of Immune system - human derived cell lines	-May not be representative -Dampened immune response -Auto-immunity against host	HCC827, NCI-H1975, HSC4, RKO PD-L1 positive cell lines were engrafted on to humanized NOG mice deficient for mouse FcγR genes to evaluate the anti-cancer effects of nivolumab [15].
GEMMs		-complex modelling - intact immune system present	-Cancers may not develop or respond in the expected way due to genetic alterations	Transgenically bred PD-1-deficient mice were used as part of this study to confirm that the administration of an anti-PD-L1 antibody suppressed tumour growth in a myeloma cell line [16].
Patient derived	Dispersed cells (ex vivo)	-quick screening - associated cell types including fibroblasts	-Short life span of cells -Immune cells unlikely to be present	-Ex vivo co-culture models assessed immunotherapy in patients with colorectal cancer [17]
	Organoid	-Able to represent the patient's TME	-Short life span -lacks vasculature and the ability make immune cells -Suitable tissue hard to source	Paired melanoma and lymph node specimens from patients with advanced melanoma formed viable organoids (90%). Treatment with pembrolizumab, nivolumab, ipilimumab and dabrafenib/trametinib matched clinical response (85%)[18].
	PDX – humanized models	-Able to represent patient's TME -Immune system present	-Cost and time consuming -Suitable issue hard to source -High rate of failure to take grafts	Partially human leukocyte antigen matched TNBC PDX cells formed tumours in humanized IL2Rγnull (hNSG) mice. Human CD45+ cells were detectable in PDXs models and anti-PD-1 antibody therapy caused reduction in tumor growth and increased survival consistent with clinical findings [19].

Atezolizumab is a humanized monoclonal antibody immune checkpoint inhibitor that selectively binds to PD-L1. It was initially assessed in combination with paclitaxel for all breast cancers which included 45% of patients had TNBC. The results from this shows promise in the phase II trial with a median overall survival of 21.3 months with atezolizumab plus nab-paclitaxel and 17.6 months with placebo plus nab-paclitaxel [23]. It was this that initially led to early FDA approval for the use, however use of this was withdrawn a couple years later after the phase 3 trial IMpassion131 revealed that adding atezolizumab to paclitaxel did not improve PFS in the PD-L1–positive population with a PFS of 6.0 months for patients who received atezolizumab and paclitaxel compared with 5.7 months for patients who received placebo and paclitaxel [24]. More recently Atezolizumab has been trialled in a phase 2 trial in combination with carboplatin for patients with metastatic TNBC (TBCRC 043)[25]. Here, PFS was increased by from 2.2 to 4.1 months which is a similar benefit to the results from KEYNOTE 355. Interestingly, patients with high TILs, high mutation burden and prior chemotherapy received greater benefit to the addition of Atezolizumab to carboplatin and those with luminal androgen receptor positive TNBC fared worse. The phase 3 trial is currently recruiting, and it will be of interest to see how this changes the landscape of breast cancer management in the future.

Avelumab, another monoclonal IgG1 antibody directed against PD-L1, is currently in the early phase of clinical investigation for use in breast cancer patients. At present there are reported phase I and phase II trials of Avelumab alone and in combination with other agents which shown promise[26–28]. Of interest the combination of Avelumab and a PARP inhibitor, talazoparib, has been reported in a phase 1b and 2 non randomized controlled trial, in patients with advanced solid tumors stratified by tumour type. The patients with breast cancer the ORR was 18.2% (95% CI, 5.2%-40.3%) in patients with TNBC; 34.8% (95% CI, 16.4%-57.3%) in patients with ER-positive, HER2 negative breast cancer; and 63.6% (95% CI, 30.8%-89.1%) in patients with platinum-sensitive, BRCA1/2 mutated breast cancer[26]. These results may suggest a niche for this combination of treatment and larger phase III trials would be needed to help define this. Avelumab has also been trialed in combination with radium 223 to specifically target patients with predominant bony metastatic disease.

Adoptive cell transfer, most commonly CAR T cells, have been of increased clinical interest due to the specificity and personalization of treatment. Here they can be used alone, or in combination with other immunotherapy agents. One such study [29] includes a description of 42 patients with treatment refractory metastatic breast cancer who underwent surgical resection of a metastatic lesion(s), isolation of TIL cultures, identification of exomic nonsynonymous tumor mutations, and immunologic screening for neoantigen reactivity. Following this, 13 patients were found to be suitable for T cell transfer and 6 patient were recruited to a II pilot trial of adoptive cell transfer of selected neoantigen-reactive TILs, with a short course of pembrolizumab. Of these, objective tumor regression was noted in three patients, including one complete response (over 5.5 years) and partial response in 2 (6 and 10 months). The time involved and cost of screening for such patients is high and further refinement of CAR T therapy is needed before it reaches mainstream adoption.

Oncolytic viruses are treatments which cause both direct tumour lysis and stimulation of an immunogenic response. They exist as many forms and can have a de novo or engineered preference for replication within cancer cells. There are many phase 1 trials of dose escalations and tolerability for viruses within the breast cancer setting, however, phase II trials are limited to a reovirus[30], herpes virus[31] and oncolytic vaccinia virus[32]. The draw of oncolytic virotherapy is the changes that are observed within the tumour microenvironment (TME) as this has been shown to induce the inflammation of the tumour microenvironment by the initiation of immunogenic cell death[33]. Together this tactic can be potentially used to sensitize otherwise refractory TNBC to immune check point inhibitors and increase response to oncolytic virus treatment [34]. Clinical studies of combinations of immunotherapies in breast cancer are therefore emerging.

Despite the advances described above, there are still many unknowns, why does immunotherapy work for some patients and not others? Are there any ways that we can improve response rates to treatment? These questions may be answered through further understanding about the tumour biology and the tumour microenvironment explored through the selection of the appreciate breast cancer model.

In vitro techniques

Utilising cell cultures make it possible to understand cell biology, tissue morphology, mechanisms of diseases, drug action, protein production and the development of tissue engineering [35]. Traditionally, cancer drug discovery started by assessment of response using a monolayer of immortalized, well characterized cell lines. They can be derived from

a number of hosts however human and murine derived are most common. These cell lines were established from aggressive primary tumours or their metastatic sites and some date back to the 1950s. Over time they have kept their malignant potential and are cryopreserved in liquid nitrogen until needed. They are at risk of contamination and changes in baseline characteristics with repeated passages such that cell lines are often replaced when the passage number exceeds 30. Some are engineered to express proteins such as GFP and luciferase which allow dynamic quantification of the cells of interest. These cell lines can be used to generate both 2D and 3D models of breast cancer within matrices of varying complexities. However, given the heterogeneity of breast cancer, there is a significant loss of generalizability of the data from these cell lines to the clinic.

Two dimensional cell culture systems

In two dimensional (2D) models, monolayers of single cell lines are grown in tissue culture plates and passaged when confluent. The advantages of this model type are the simplicity, reproducibility, and low cost. This is of particular importance in high throughput screening. The access to these cell lines are widely available and there have been a number of reviews summarising the key characteristics and behaviour of the these established cell lines [36–38]. To assess immune function 2D cancer cell cultures can be enriched or co-cultured with immune cells and immune cell mediators which can simulate the TME. Immunogenic cell death is a feature that lends well to be studied through 2D cell culture systems. Here, co-cultures of cancer cells and immune cell of interest can be co-cultured[39–41]. Cancer cells can then be stimulated to undergo immunogenic cell death and the phagocytosis, effect and maturation, activation of the immune cell of interest can be assessed through flow cytometric measure of cell surface markers or ELISA of cytokines such as HMGB1, IL-17 and type 1 IFN [42,43]. To simulate the heterogenous make-up of the TME, co-cultures can be made more sophisticated through the addition of numerous cell lines. In our laboratory, lymphocytes are isolated from waste buffy coats before co-culturing with monocyte derived macrophages and 2D cultured human derived TNBC cells. We have shown that within this co-culture mixed lymphocytes show activation when exposed to an oncolytic virus treatment[33].

The main advantage of 2D models is the effect on cells can be directly observed and variables can be controlled to confirm causality rather than association. However, as the external conditions in which cells are grown does not mimic the natural host's system, behaviour of the cells may be different to those seen in vivo and changes in cell morphology, due to adherence to the bottom of plastic plates, cause cells to be longer and flatter which changes their exposure to the culture medium. Additionally, an assessment of hypoxia is not possible as monolayers receive a uniform homogenous amount of nutrients that is replenished with each passage [35]. Furthermore, monolayers of cell lines are often grown in isolation and therefore do not recapitulate the innate tumour cell heterogeneity and tumour microenvironment as they lack the cell-to-cell interactions, tissue structure and surrounding cellular components of the tumours such as fibroblasts, macrophages, and other immune cells. Traditionally, researchers have moved straight from 2D models to in vivo assessment of novel therapeutics. However given these limitations, this may not be ethical and incurs financial and time expenses, therefore there is a growing interest in advanced cell culture techniques which involve the inclusion of a structure for cells to adhere to, co-culture with non-epithelial cells or a diffusion gradient created through microfluidics.

Three-dimensional cell culture systems

Three-dimensional (3D) cell cultures systems have developed in response to a growing awareness of the importance of the interactions between tumour cells and the extracellular matrix (ECM) they are suspended in. Therefore, although 2D cell culture is useful in high-throughput screening of drug in plates to assess sensitivity to differing agents, 3D cell culture may be more useful in drug discovery and can potentially lessen the importance of in vivo work. The extracellular matrix consists of a milieu of different protein structures and growth factors that facilitate interconnections between cells. Alterations in ECM composition may influence drug response through altered drug availability, expression of drug targets, or changes in cellular defence [44]. The advantages and disadvantages of 3D over 2D cell culture systems is summarized in table 1 in the paper by Kapałczyńska et al [35]

Importantly, 3D culture allows the possibility of co-culturing cancer cells with other cell types within an infrastructure that can reproduce the challenges of delivering treatments to the TME. For example, the co-culture of cells of either different type or origin can allow for the assessment of cross talk between these cells. Arrigoni et al., describe a systematic review of breast cancer metastasis towards bone and how the interaction between the bone microenvironment and tumour cells can be stimulated by co-culturing bone and cancer cells in numerous ways. This included incubating cells in tumour conditioned medium, directly mixing bone

and cancer cells, or allowing cancer cells to permeate an artificial bone membrane and track into the monolayer of bone cells [45].This concluded that advancement in understanding of bone metastasis was only possible because of the precision and control of co-culture in vitro systems which would not have been possible in an in vivo system alone.

Three-dimensional cell cultures have also started to expand into the world of addressing immune system modelling. For example, an immunogenic 3D breast cancer model was recently described using MDA-MB-231 cells and patient derived immune cells cultured at ratio of 1:1 [46]. The addition of patient-derived cells more accurately represents the TME the crosstalk between both cell types to be studied for up to 10 days, as well as the assessment of antitumour immune responses to immunotherapies. These experiments have shown differences in response between the tumour cells alone, tumour and immune cells and immune cell alone groups (unpublished observation) which support the need to use advanced cell culture techniques when exploring immunotherapeutic efficacy. Tevis et al. generated a TNBC 'heterospheroid', containing breast tumour cells and macrophages embedded in a collagen gel [47]. This model displayed increased secretion of anti-inflammatory IL-10, suggesting that the macrophages adopt a more M2-like phenotype upon co-culture with MDA-MB-231 cells. Furthermore, this model exhibited resistance to paclitaxel treatment in comparison with MDA-MB-231 monoculture spheroids.

Although there are several 3D cell culture systems in the literature, they can be broadly divided into scaffold dependent or scaffold independent. Scaffold independent systems rely on the self-aggregation/organization of cells when placed in specialized culture plates or media. For drugs where hypoxia is important, spheroids can be created with their own ECM and grown to a size where they demonstrate a hypoxic gradient within its core. The main disadvantage of spheroids and other non-scaffold systems is challenges in the reproducibility of these cell models in terms of size and culture conditions. An exploration of the substrate in which breast cancer cells are grown have led to the development of hydrogels. One particular model describes the use of an alginate/Matrigel hydrogel to study invasion of TNBC MDA-MB-231 cells. Malignant cellular morphology such as invadopodium, actin-based protrusions of the plasma membrane through which cells anchor to the extracellular matrix was demonstrated [48]. This feature is thought to be key in the development of metastatic potential of malignant cells and may provide insight into how this mechanism may be addressed with cancer therapies. Interestingly, these 3D models can also be used to model the inflammatory microenvironment where different cell types can be incorporated e.g. adipose-derived stromal cell [49] thus also enabling obesity in breast cancer to be investigated [50].

In essence 3D models have a potential to provide small, controlled environments to repeated assess with immune cells for a short duration of time. Within these environments the mix of cell lines, cell types and even tumour microbiome can be altered to further mimic the heterogeneous nature of the TME. These models can be made more sophisticated using microfluidic assays.

Microfluidic Assays

Microfluidics assays (MFAs) are a branch of three-dimensional models that are intended to recreate *in vivo* microenvironments *in vitro*. These MFA devices or chips can vary in design but are commonly made from transparent moulded or engraved materials suitable for cell culture to allow the imaging and real-time tracking of cells introduced into the MFA devices using confocal microscopy. They may focus upon the internal dynamics and structure of the vessel lumen or on the external PV microenvironments but are based on the application of fluid flow to channels through the device. MFAs have been adapted to form 'organs on chips' by growing micro vessels in gels that mimic the extracellular matrix (ECM) of various tissues or organs [51] .They are now increasingly being used to look at events within and close to micro vessels in defined environments like gels *in vitro*. They have already been used to extensively monitor both the intravasation and extravasation of cancer cells through micro vessels [52] and are now increasingly being used to explore the role of immune cell subsets with one another and micro vessels [53,54].

There are typically two kinds of MFA models, those that use a precast pattern or network, in which endothelial cells are seeded in to coat the exposed surfaces and models that rely on vasculogenesis, in which the micro vessel network is formed by mixing endothelial cells in ECM gels. Bischel and colleagues used a precast device to generate micro vessels within a collagen/Matrigel hydrogel. Briefly, the channels of an MFA device were filled with polymerised ECM gel. Media was then pumped through this until it had re-created channels through the gel, and then lined these with HUVECs to produce vessel-like structures [55]. The Kamm lab[56] also used HUVECs and a gel to form a

microvascular network in an MFA device. With their model, the microvascular network could also be formed in the presence of human lung fibroblasts (HLFs) [56,57]. Here, the MFA device consisted of 3 parallel channels, the centre channel, designed to hold the hydrogel, was separated from the outer media channels by trapezium posts. These held in the pre-polymerised hydrogel and stopped it leaking into the media channels. To produce a microvascular network, HUVECs and HLFs were seeded in a co-culture in a fibrin gel. Then over a 4/5-day period they formed vessel-like structures with lumens that span the central gel region and connected the flanking media channels[56].

Microfluidic systems are often used to access flow. In the immunotherapy environment the bystander effect of oncolytic virus therapy is of interest. Lee et al designed a link system of two microfluidic-based models which mimic the delivery of oncolytic viruses through the blood stream to cancer deposits. The flow condition used were similar to flow level that can generate the interstitial flow and shear stress for simulating the in vivo microenvironment and the dispersal of oncolytic virus within the system can be observed. However, this system was limited by the lack of immune cells with the assay [58].

Other groups have adapted these MFAs to investigate the 3D interaction of human monocytes/macrophages with human tumour cells. This has shown how the model can be used to monitor the interaction between human tumour cells and immune cells - and identify the mechanisms of their interaction. In one such example, a single media channel was lined with HUVECs and introduced macrophages and A549 lung carcinoma spheroids into the collagen gel in the middle channels. Prior to embedding in the gel macrophages were preconditioned into a M0, M1, M2a, M2b and M2c phenotype, this was done by treating macrophages derived from buffy coat isolated monocytes for 24 h. Macrophages were left untreated (M0), grown with LPS and IFN-γ (M1), IL-4 (M2a), human IgG and LPS (M2b) or IL-10 (M2c). Macrophage infiltration towards tumour spheroids and the effect of differences in macrophage phenotype on dispersion for the A549 aggregates in the gel were observed. Of note, culturing with macrophages of an M1 or M2b phenotype showed the greatest dispersion of tumour spheroids, these effects were seen with and without HUVECs lining the media channel. Dispersion of tumour cells was seen to be promoted by contact dependent mechanism involving CD11a and CD11b. When there are blocked macrophages or its receptor, ICAM-1 on A549 cells, a significant decrease in the dispersion of tumour spheroids is observed [59].

A more recent study by the same group showed that human monocytes infused into MFAs extravasate through the micro vessels into the perivascular region. Here the role of the CCR2 signal is correlated to relate to tumour growth and invasion through promotion of angiogenesis, recruitment of M2 like macrophages and suppression of CD8+ T cells. It is reported that a higher number of inflammatory, $CCR2^+$ monocytes were able to extravasate through the vasculature than those which were $CCR2^-$. Moreover, following extravasation $CCR2^+$ monocytes begin to upregulate MRC1. However, this was not linked to extravasation, as monocytes that were seeded directly into the fibrin gel, with HUVECs and HLFs also displayed the same levels of MRC1 upregulation [60]. Studies like the above have since led to the development of advanced MFAs to study human breast cancer cell extravasation into an actively secreting bone microenvironment generated by embedding human bone marrow-derived mesenchymal stem cells (MSCs), endothelial cells (ECs) and osteoblast-differentiated cells (OBs) using a gel system. The ECs form vasculature, whereas MSCs and OBs create a bone microenvironment. Cancer cells introduced in the vessel extravasate into the organ-mimicking gel which can be used as a drug screening platform[61].

Microfluidics can also be used to test drug sensitivity to treatment. In a novel model involving a co culture of MDA-MB-231 cells with HMEpiC cells, cancer cell migration was assessed through assays of IL-6 and CK14. In this model, treatment with anti-cancer agents paclitaxel and tamoxifen were shown to decrease migration.

Although there are several advantages to in vitro models, namely the convenience, reproducibility, lack of animal work and potentially cost savings, the use of a host allows the exploration of a medications effect on the whole-body system. This is particularly important for immunotherapies where key benefits of treatment are to induce systemic anticancer effects through stimulation of the immune cascade.

In vivo models

The complexity of the human vasculature and drug clearance is best assessed within a living model and in vivo studies are felt to be a required "gold standard" before clinical trials.

In vivo breast cancer models can be formed through genetic modification, spontaneous/chemically induced tumouriogenesis or implantation of human, animal derived (predominantly murine) or human cell lines. There are significant differences between human, humanised and murine breast cancer cell lines notably in relation to the tumour environments compared to patient derived xenografts[62]. In particular, the tumour immune interactions (both at the site of the tumour and systemically) can be observed, and additionally the stromal components of the tumour (e.g. fibroblasts) are of tumour origin. These differences mean that the true nature of immunotherapies may not be best exposed in their models.

Immunocompetent animal models of breast cancer are limited. Murine breast cancer can either arise sporadically and spontaneously in fully immunocompetent non transgenic mice mimicking the de novo presentation of human breast cancer, be induced through inoculation of a known murine breast cancer cell line or arise spontaneously in mice that been engineered with transgenic genetically engineered mouse models (e.g. GEMMs.). Below is a discussion of the use of patient derived and genetically engineered mouse models.

Genetically Engineered Mouse Models

Genetically engineered mouse models are created by random integration of a transgene into the genome, which results in gene overexpression in transgenic mice, gene deletion in conventional and conditional knock-out mice or targeted insertion of the transgene in a specific position known as knock-in mice [63].

Conventional knock-out mouse models are advantageous due to enabling the study of specific oncoproteins and allowing the analysis of the interactions between protein domains and mutations and how they contribute to the progression of disease. The immune system remains intact, and different stages of tumour progression can be studied, including metastatic disease. Regarding immunotherapy an increase in the mutational burden can lead to the formation of neoantigens that can be recognized by immune cells [64] and this can lead to the evolution of the anti-cancer immune response, and studies of how this may affect the effectiveness of immunotherapy. Limitations arise with regards to the process being time consuming and expensive, as well as the consistency of the models being executed, this is due to the knocked-out genes being switched off, in all cells, at all times. Knock-out mice also do not truly represent human tumour development, due to the mouse microenvironment.

Conditional knock-out mice are models where chemically generated transcription factors or deletion of tumour suppressor genes can be controlled, with regards to when the target gene is turned on or off[65]. This gives advantage over conventional knock-out models due to the decreased risk of the mice displaying developmental abnormalities, which increases the consistency of models being executed with aids in reproducibility. A summary of genes which have been targeted in breast cancer models are shown in figure 1 and table 2.

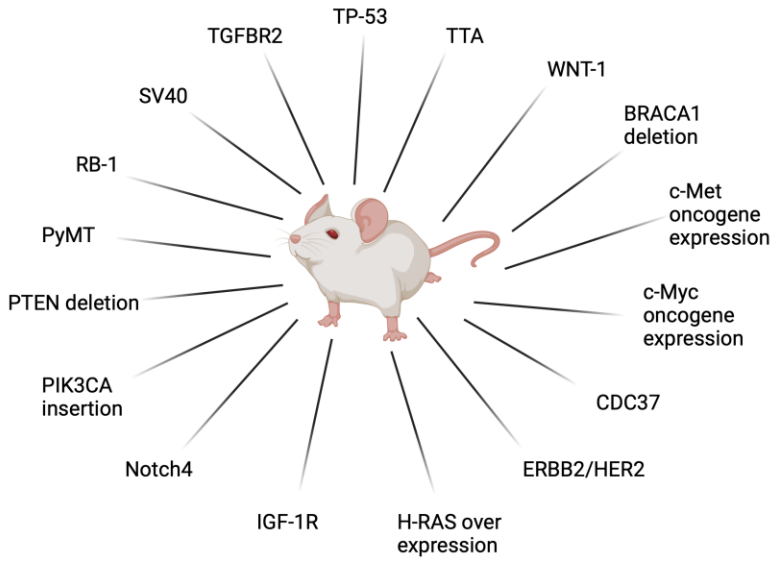

Figure 1: The spectrum of genes that can be engineered to be over or under expressed within mice to form GEMMs. Genes can be deleted singularly or in combination.

Table 2: A selection of published transgenic mice strains available in breast cancer and their features.

Gene	Strain	Model name	Features	
BRCA1 deficient	Mixed	BLG-Cre;Brca1F22 – 24/F22 – 24;p53+/−	Basal ER negative, HER2 +	66
expression of c-Met oncogene	FVB	Met^{mut} (M1248T/L1193V)	Met receptor expression	67
Expression c-Myc oncogene	FVB	MTV/MYC fusion gene		68
Over expression CDC37	Mixed		Poorly metastatic	69
ERBB2/HER2	FVB	FVB/N-Tg(MMTV-ErbB2*)NDL2-5Mul mouse	Expresses PDL-L1 and responds to PD-l1 inhibitors	70
H RAS over expression	FVB	MMTV-v-Ha-ras	Salivary and lymphoid and mammary tumours	71
IGF-1R	Mixed		Produces salivary gland tumours Weakly ER/PR +	72
PIK3CA insertion	FVB	Pik3ca(H1047R);MMTV-Cre mice	Multiple tumour subtypes	73
PTEN deletion	C57/BL6	Mammary specific PTEn deletion		74
PyMT	FVB	Eg MMTV-PyMT FVB/NJ strain uses MMTV-LTR to drive expression of PyMT	Loss of ER, variable overexpression of HER2. Immune cell infiltration is high. Lung mets common.	75
RB1	Mixed	MMTV-Cre:Rbfl/fl	Latency of 18.4 month. ER negative, luminal B or basal like tumours. Lung metastases in 50%.	76
Sv40	FVB	PSBP C3(1) 5′ flanking sequence to drive expression of Tag	Invasive ductal cancer from 16 weeks age. 15% lung mets. Loss of ER. Responds to 1L-12 immunotherapy	77
TGFBR2	C57/BL6	Truncated transforming growth factor beta receptor 2	Invasive cancers with lung mets	78
WNT 1	FVB/mixed	MMTV-Wnt1	2 subtypes reported; early (more cellular) and late (more vascular) tumours.	79
TTA	FVB/C57/BL6	Tet-op-Esr1MMTV-tTA/tet-op-SV40-TAg	ER+ adenocarcinomas latency of 11 months. Lymphocytes present in TME.	80
deletion of p53 and Brca1	FVB K14-cre mice	K14-Cre; p53f/f Brca1f/f	human basal-like breast cancer Propensity to have immune cell infiltrates. Sensitive to carboplatin and paclitaxel.	81
Amplification of MYC and deletion of PTEN		Myc;Ptenfl RosaLSL-Myc/LSL-Myc;Blg-Cre strain with the Ptenfl/fl-conditional knockout	TNBC histology Complex tumour immune environment described	82

Genetically engineered mouse models have contributed to the understanding of the immune systems role in the early development of breast cancer where the use of the mouse model of mammary tumour progression that expresses the Polyomavirus middle T (PyMT) and Cre recombinase (Cre) in a doxycycline-inducible fashion (MMTV-MTB/TetO-MIC) revealed the importance of STAT3 in creating the immunosuppressive environment which enables the immune system evasion in the early stages of tumour growth and metastatic breast cancer. In this model the conditional STAT3 allele is deleted in the mammary epithelium through induction with doxycycline. Stat3 deficient mice were found to have a profound delay in mammary tumour onset and the tumours that emerge did not reach their full metastatic potential. Furthermore, an increase in activated T cells and macrophages was observed post mortem[83].

Genetically engineered mouse models can also be used to ascertain response to check point blockade therapy. In a study by Hollern et al [84], two different GEMMs: Tp53−/− tumour syngeneic transplant derived cell line (T11) and a cell line from a K14-Cre;Tp53f/f; Brca1f/f tumour (KPB25L) were used to identify genomic signatures which suggest treatment response or resistance. Through this they developed new mutagenized models for studying immunotherapy in TNBC as conventional GEMM mammary tumour models were resistant to immune check point blockade and possible due to low tumour mutational burden. They correlated these

with findings within human breast and melanoma models and found higher representation of B cell populations seemed to predict response. Further to this, they described a B/T cell sub population which has the potential to be used as a biomarker to suggest response to treatment. B cells are an increasing area of interest in immuno oncology, and it will be interesting to see if these studies will provide translatable clinical effects in the years ahead.

Patient Derived Xenograft Models

Patient Derived Xenograft (PDX) models are generated using human tissue samples, which are taken directly from a tumour biopsy, in a sterile environment. The sample taken is divided into fragments and inserted directly into the cells of a highly immunocompromised mouse - this mouse is termed the first passage. Once established, the sample is then transplanted into multiple mice, as a subsequent passage and so forth.

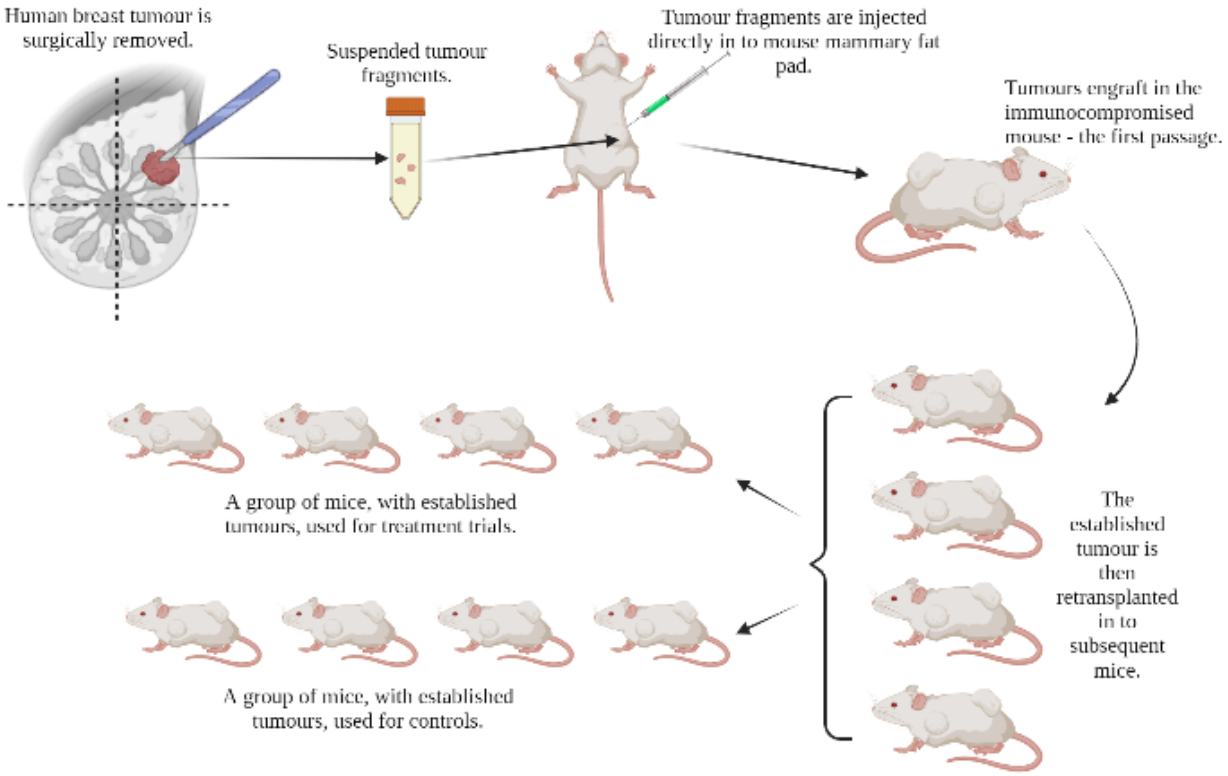

Figure 2 legend: The creation of a PDX model. Cancer containing tissue is removed en bloc and fragmented but not digested to maintain tissue structure. These are then implanted into an immunocompromised host and following tumour engraftment, are repassaged until tumours stably reproduced. A number of PDX lines can be used in series to provide a heterogenous variety of test subjects. Mice of the same strain are used to provide the control.

A large amount of PDX models can be generated at one time, which enables numerous medications to be screened together. Research can be carried out to find the most effective treatment, with the best response rate, at the optimum dose, this also allows study into any treatment resistance [85]. Patient derived xenografts allow for multiple biopsies at different points of treatment, this means that treatment can be specifically adapted, and genetic changes can be studied throughout. Most breast cancer PDX models are orthotopic which means that the primary tumour site from the human, is imitated within the mouse, for example, a TNBC tumour sample would be inserted into the mouse mammary tissue, by injecting tumour cell suspension directly into the mammary fat pad. Recently, this model has been improved by injecting PDXs directly into the mammary duct, enabling interaction of tumour cells with the mammary gland and formation of heterogenous tumours that histologically mimic the original patient tumour[86]. Utilising this method also increases the chances of mammary tumours metastasising to the same site as observed in the patient. Importantly, implanting human bone into immune compromised mice before intra-ductal injection of PDXs provides a human specific site for which PDXs can metastasise to and also results in human specific haematopoiesis generating human specific immune cells, however, the functionality of these cells remains to be determined [86].

What is particularly of interest is the validation of treatment responses of PDX models in comparison to clinical response observed. Pettersen et al described established a PDX model from fragments of patients with breast cancer and were able to observe human tumour cells within the implanted tissues [87]. Furthermore, the response to paclitaxel treatment in in the animals correlated with observed clinical responses suggesting if the implant is successful this could be used to assess and predict treatment in a proactive manner ahead of when a patient may need it.

Advantageous in comparison to other models, as the sample comes directly from a human, it maintains the genetic heterogeneity of the original tumour, as well as histological structure of the patient. However, PDX models are expensive and time consuming – sometimes requiring months to establish adequate tumour engraftment, the validity of a biopsy could be doubted on its relevance to human structure, due to the timescale required. Due to these limitations, PDX models are only able to give a part representation of the development of the tumour and its microenvironment, at present. This means that further study into the long-term effect of treatments is difficult.

Other limitations arise, due to the use of immunodeficient mice, though this is currently the best model to represent a human tumour, without rejection, immunotherapy cannot be studied due to the lack of immune system[85]. It is already understood that the immune system can be targeted to help support tumour eradication. Further study is needed to replicate the human immune system, within PDX models, without the risk of rejection of the sample. A closer representation of a tumour's microenvironment could then be simulated, with regards to the immune system involvement. Immunotherapy could then be studied and tested on PDX models to possibly support combination therapies which are currently already routinely used in other types of cancers.

Humanised Patient Derived Xenograft models

The introduction of human hematopoietic stems cells in NSG or BRG mice has led to the creation of a hybrid "humanised" model. In this process mice are first irradiated with whole body gamma irradiation between 5-10 weeks of age, and subsequently human CD34+ stem cells are intravenously injected and allowed to engraft. This is monitored via flow cytometry at around 10-12 weeks where successful engraftment is considered when mice have more than 25% human CD45+ cells in circulation [88]. Once established human tissue can be introduced as per figure 2. As these mice are now as semi-immunocompetent host, their immune can be assessed[89] and immunotherapies can be introduced. What is particularly promising about these models is the suggestion that the TME can be preserved. Morton et al., describes that human immune cells were able to infiltrate engrafted head and neck tumours within a humanised mouse model. Furthermore, these cells were able to induce lymphangiogenesis and sustain the original gene expression profile of the PDX [90].

Humanised models show promise for investigation of immunotherapy treatment with checkpoint inhibitor therapy has been assessed in humanized NSG mice for bladder cancer[91], hepatocellular carcinoma (HCC) [92], melanoma[93,94], non-small-cell lung cancer (NSCLC) [95,96], autologous renal cell carcinoma (RCC) [97], and TNBC [94,98]. In a humanised nasopharyngeal cancer model with NSG mice, Liu et al have interestingly observed matching clinical and preclinical responses to the combination immunotherapy of nivolumab and ipilimumab with significantly increased IFN-γ and IL-6 production and decreased the CD4/CD8 ratio in a humanised PDX model compared to their non humanised PDX model[99].

A TNBC PDX-engrafted HSC-humanised NSG mouse model was designed to show TNBC patients positive for programmed death-ligand 1 (PD-L1) can benefit from the anti-PD1 immune checkpoint inhibitors atezolizumab or pembrolizumab in combination with chemotherapy [100,101]. In these studies, some mice had reduced tumour growth upon treatment with the anti-PD1 pembrolizumab or nivolumab, while no effect was observed upon anti-cytotoxic T lymphocyte antigen 4 (CTLA-4) ipilimumab treatment.

Furthermore, these humanised models can confirm the checkpoint receptor expression which may result in further treatments to be targeted towards a certain population. An example would be the results of immune checkpoint profiling in a group of humanized breast cancer mice which has shown co-expression LAG-3/PD-1/TIM-3[102]. Perhaps this will form the foundation to trials to investigate the use of a LAG3 inhibitor and PD-L1 inhibitor in breast cancer as has recently been approved for melanoma. Additionally, as a reliable ER positive model has been difficult to conventionally develop and there has been great interest in humanised breast cancer mouse models in the ER+ group and a number have been described. One such is the immune-humanized ER+ model where the HCI-

013 PDX line (a metastatic, endocrine resistant ER+ model of lobular breast cancer)[103].

Limitations of PDX models revolve around costs and difficulties with engraftment - more aggressive breast cancers have high engraftment rate [104]. In one centre the overall 'take rate', defined as PDX growth for at least two generations, was only 29%. Primary tumours were found to be more challenging to engraft (25% of 102 attempts) than metastases sites (36% of 50). ER positive PDXs were the most difficult to develop, with a take rate of 9% for primary ER positive tumours ($n = 32$ attempts) in contrast to TNBC with a take rate of 58% for primary tumours ($n = 12$ attempts) [105]. Despite this, these models have started to help bridge translation research the gap between bench and bedside [106].

There are concerns about the number of animals needed for the generation of PDX models with protocols often requiring multiple animals per patient and low engraftment rates. Organisations such as the National Centre for the Replacement, Refinement and Reduction of Animals in Research (NC3Rs) are therefore supporting ex vivo technologies as alternatives to our dependence of animal models as gold standards.

Patient derived explant models (ex-vivo models)

The use of ex vivo based models that use fresh surgically resected tumour or biopsy material has seen a resurgence since their first use in the 1950s.

In order to better replicate the effects of drugs within the patient, that aren't seen within cell line models, the development of drug screening assays that utilize patient material has begun to take off, with multiple different approaches now been taken to develop models that will predict drug resistance, biomarker discovery and drug development. By using patient derived material, this is the next step in tailoring personalized medicine, allowing data to be cross-referenced with the diagnostic and patient outcome. Currently there are a range of approaches to patient derived material in an ex vivo setting which are summarised in figure 3 and described below.

Preserved microenvironments

Ex vivo assessment using breast cancer specimen using a perfusion bioreactor has been shown to maintain both tumour and immune cell viability for 7 days. Using 2mm^3 cut fragments, cultured between a collagen scaffold, these are perfused at a constant flow rate with supplemented culture medium. Tumours were treated with Fulvestrant, Pertuzumab, anti PD-L1 and anti-CTLA4 by adding to the culture media. Samples were fixed and embedded before staining was performed on sections for assessment. Higher cell viability was seen is perfused culture vs static cultures as measured through negative caspase 3 staining. Fulvestrant treatment on ER+ tumours, significantly reduced epithelial cell viability compared to untreated controls. With an effect also seen when HER2+ tumours were treated with Pertuzumab. Treatment with anti PD-L1 and anti-CTLA4 in 3 TNBC tumours showed a significant decrease in cancer cell viability after 7 days of treatment hen the controls of lymphocytes and normal breast tissue remained unaffected [107].

In a separate study, looked at the paclitaxel treatment on using 200um thick breast tumour slices [108]. These were then cultured for 24 hours before the slices were treated with vehicle or paclitaxel for a further 48 to 72hrs. Samples were then fixed embedded and stained for cell death markers and proliferation. Paclitaxel treatment on explant cultures did not induce high levels of cell death during the experiment timeframe, but an increased uptake was seen in tumour cells, it was suggested that cell death from paclitaxel would have occurred, but this was not observed due to the time limits on the explant culture. Treatment of samples with another microtubule inhibitor, Vincristine, did elicit a response. This indicates that in some instances, some commonly used standard care drugs, may not be suited to the ex vivo environment.

Response to drug screening using patient material has also been seen in NSCLC. This method, using fresh surgical tumour tissue, adds a pre recovery phase where the tumour is cut into 2-3mm^2 pieces and cultured for 16-20 hours prior to treatment. These tumour pieces are then transferred for culture with the therapeutic compounds for 24 hours before being fixed, embedded and sectioned for analysis through IHC/IF. Using cisplatin, this was shown from 26 patients, the response of NSCLC to Cisplatin using this assay saw a link to patient outcome. Using cPARP staining as a measure of cell death to the highest levels of Cisplatin, they determined a cut-off for sensitive and resistant tumours. The sensitive samples identified were shown to correlate with patient survival, although there was no minimum follow-up period for the patient data [109].

Patient derived organoids

Patient derived organoids are 3D reconstruction of patient derived tissues within an ex vivo environment that tries to stimulate the environment

in vivo. The advantages of this over 3D scaffold systems is that the organoids can self-renew and differentiate into different cell lineages. However, they lack a vascular system and therefore can only be sustained for a limited number of passages [110]. The diversity of cell milieu within the organoid in both the heterogeneity of the cancer cells suggest that this may provide a useful model to screen for drug sensitivity prior to treatment. More over the receptor status of over 100 cases of breast cancer organoids continued to be well matched in histopathology, hormone receptor status, and HER2 expression to their original tumours [111]. In support, the fidelity of cell lines, PDX, PDOs and genetically engineered mouse models were assessed using and AI assisted programme which suggested that general, genetically engineered mice and PDOs reveal higher transcriptional fidelity than PDX and cell lines[112].

Unfortunately, culture mediums and the lack of vasculature on formation of the organoid means that immune function can be difficult to ascertain. Some groups have published protocols to co-culture organoids with lymphocytes and CAFs [113,114], but these have yet to be investigated in trials. There have however, been successful reports of PDO being use to assess the specificity and enhance efficacy of CAR-T cells [115].

A similar 3D concept can be seen in within the development of patient derived spheroids. In patients with several histological subtypes check point blockade can be shown to have similar response in a PDOT ex vivo microfluidic based model. Here authors also despite cytokine profiling within this model which has led to a suggestion that those with immunosuppressive cytokine expression (CCL19/CXCL13) had a decrease in clinical PFS survival. This is of particular clinical interest as outcome prediction tools to allow more personalised treatment can help clinical weigh up the risks and benefits of certain treatments [116].

Dispersed methods

Keeping the tumour environment intact does have its advantages in that the changes can be seen in situ. However, if the same drug response is also given once the tumour microenvironment is dispersed and still correlates to the patient response, then this is a potentially more powerful tool. There is greater scalability of drug screening assays by removing the limitations on the number of compounds tested, while response to compounds can be seen using a fraction of the cells used in organoids and preserved microenvironments. Although well suited to haematological cancers where promising results have already been seen [117] this is increasingly being used within solid tumours[118] .

Unlike ex vivo methods that look to preserve the tumour microenvironment, assessment of the dissociated tumour microenvironment allows for greater scalability of drug screening assays. Although these models may lack the environmental aspects, they retain the heterogeneity of cells. Dispersed models are well suited for drug discovery screening assays, through analysing the effects of the drugs on the healthy and the cancerous population. Where multiple mutations exist within the tumour cells, this can also be picked up from large-scale screening platforms.

One study that used imaged based analysis of a single cell population in AML showed that 15 out of 17 patients had an overall response when using imaged based analysis to guide their treatment compared to 4 of 17 when compared to their previous treatment given[117]. It is however, tricky to stimulate the effect of the immune system on single cell populations in culture, but could perhaps screen if patients samples are positive for PDL1, LAG3 and other check point markers and therefore help guide which patients would benefit from immunotherapy treatments.

With the breast cancer setting ex vivo work is of increasing interest in both the diagnostic and therapeutic field. A recent proof of concept study to assess an novel ex vivo anthracycline sensitivity assay revealed that 75% patients had matching assay and clinical MRI responses to anthracyclines. A similar study assessing screening for cisplatin and docetaxel sensitivities have also been described [119]. Although the sensitivity and specificities have the potential for further refinement this is a promising use of ex vivo of ex vivo screening which would allow therapy to be targeted [120].

Challenges with patient derived cell lines

Despite the translational benefits of working with patient derived material, there are multiple challenges associated with it. The time it can take from the sample being resected, processed by histopathology and then being made available for processing in the lab, can affect the overall viability and quality of the sample with environmental conditions not being kept optimal. Even if conditions are kept optimal, burning/scarring from surgical procedures used to resect tissue can greatly hinder the viability of the cells, leading to inter patient variability between samples.

Biobanks hold an increasing number of viable cryopreserved samples, many accompanied by clinical data. As such, these may be a great source of retrospectively validating assays, but work still needs to be undertaken to understand the effects of cryopreservation on the cells and if this influences any changes in the drug response. Each assay has its own limitations that have still to be defined, such as the quantity of tissue needed, the time each assay is run and the reproducibility within the assay itself.

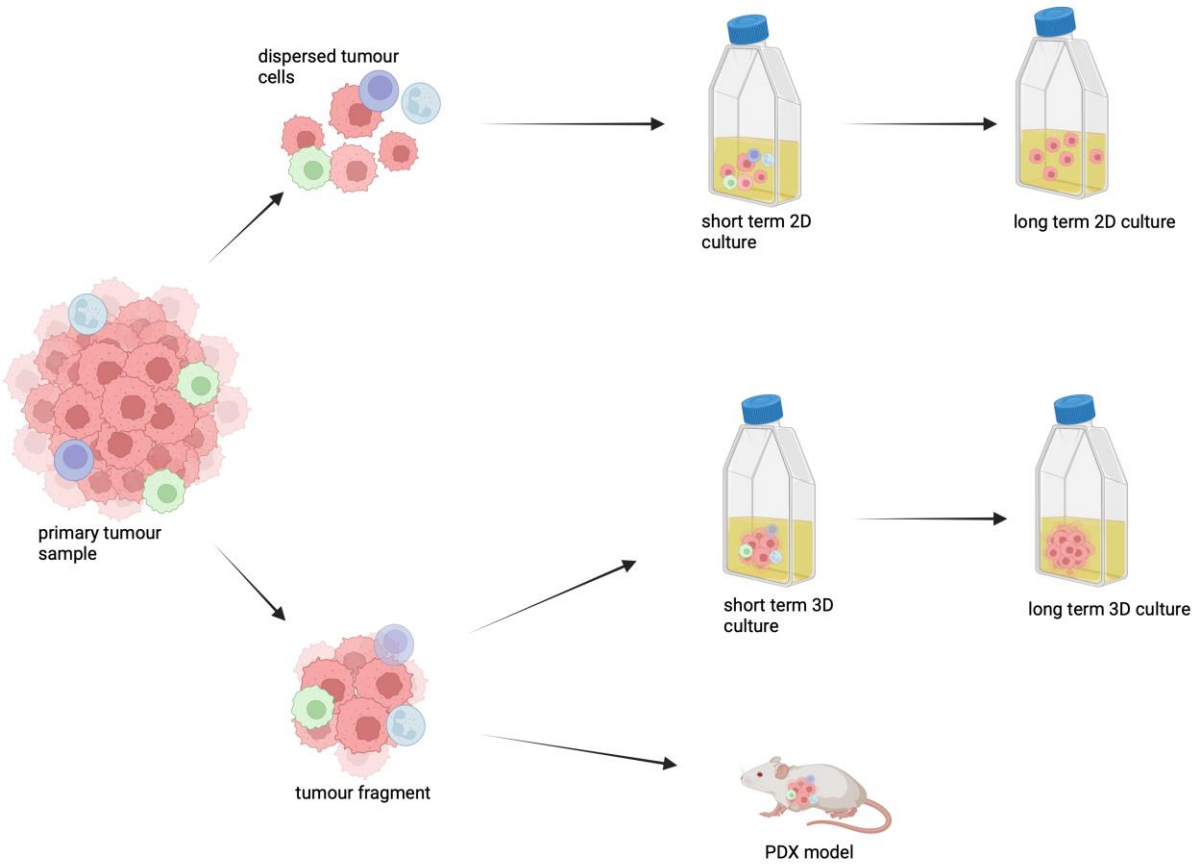

Figure: 3 Use of patient derived cells in breast cancer models. Patient derived tumour cells contain a milleu of cell types. A short-term model of the immune make up of tumours can be derived for dispersed immune cells. However, culture mediums are not able to retain immune cells. Alternatively primary tumours can be divided into fragments which can then be cultured as organoids or implanted into humanized mouse models. Organoid models allow for more complex TME modelling and assessment of penetration, but again become deplete of immune cells over a short duration of time. PDX models are an attractive alternative however studies may be limited by cost and poor engraftment rates. Created with BioRender.com

Conclusion

The overarching aim of a preclinical model is to try and simulate the complexity and heterogenicity of a patient's TME, but yet still be consistently reliable and reproducible. This is a tall ask, and yet, models may "make or break" a treatments success in the clinical trial setting. The development of Tamoxifen as an anti-oestrogenic breast cancer treatment is a good example of this. Originally developed as an effective "morning after pill" in laboratory rodents, it was found to be a poor contraceptive in humans. Additionally, it was also noted to have an anti-oestrogenic effect in rats and primates, however a pro-oestrogenic effect in mice [121]. The uncertainty of whether this treatment would be effective for breast cancer patient was anticipated, and yet, now this hormonal treatment is one of the backbone treatments for ER positive breast cancers.

Likewise, the widely used immunotherapeutic drug Pembrolizumab, was discovered when looking for a drug to treat autoimmune disease [122]. These coincidental discoveries compel us to ask whether the challenge we face as scientists is picking the right model or have a general knowledge of the body to the extent that you can see potential between disease sites. The landscape of breast cancer treatment has changed significantly over the last 50 years. Although we see improvements in survival because of patients being diagnosed and

treated early and the availability of treatment from a larger pool of drugs, we also see a difference in history of presentation such as late relapses and an increase in unusual sites of metastases, eg brain metastases.

The flexibility of models to adapt to these changes have allowed continued development of novel treatments. However, the dream model would allow personalization and dynamic testing of patient cells and the use of ex vivo breast cancer screening of drugs is of significant interest at present. There are signs that ex vivo testing can be used to screen responses to chemotherapy however we are yet to see if this can be extrapolated to immunotherapy. This review highlights the currently available models and their potential to stimulate an environment suitable to evaluate the immune system in breast cancer and paves the way to the development of future breast cancers studies.

Conflict of interest

"The authors have no conflicts of interest to declare."

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