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Steroid Hormone Receptor Positive Breast Cancer Patient-Derived Xenografts

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Abstract The vast majority of breast cancers are positive for estrogen receptor (ER) and depend on estrogens for growth. These tumors are treated with a variety of ER-targeted endocrine therapies, although eventual resistance remains a major clinical problem. Other steroid hormone receptors such as progesterone receptor (PR) and androgen receptor (AR) are emerging as additional prospective targets in breast cancer. The fundamental mechanism of action of these steroid receptors in gene regulation has been defined mainly by several breast cancer cell lines that were established in the late 1970s. More recently, breast cancer patient-derived xenografts (PDX) have been developed by multiple groups at institutions in several countries. These new models capture the large degree of heterogeneity between patients and within tumors and promise to advance our understanding of steroid hormone receptor positive breast cancer and endocrine resistance. Unfortunately, steroid hormone receptor positive breast cancers are much more difficult than their receptor negative counterparts to establish into sustainable PDX. Herein we discuss the derivation of steroid hormone receptor positive breast cancer PDX, several pitfalls in their genesis, and their utility in preclinical and translational steroid hormone receptor research.

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Introduction

Breast cancer remains the most commonly diagnosed cancer in women and the second leading cause of cancer related mortality in the USA [1]. Breast cancer has traditionally been divided into distinct histopathological subtypes based on expression of estrogen receptor (ER), progesterone receptor (PR), and human epidermal growth factor receptor 2 (HER2). Breast cancers devoid of all three markers are classified as triple negative (TN). More recently, breast cancer has been categorized into molecular subtypes based on gene expression profiling. The original and most widely used molecular panel utilizes 50 discriminator genes, termed PAM50 [2, 3]. This divides breast tumors into five groups: two ER positive groups termed Luminal A and Luminal B, and three ER− groups termed HER2+ER−, basal-like, and claudin-low [3]. The two luminal ER+ subtypes constitute 70–75 % of all cases. Luminal tumor growth is driven by the mitogenic actions of estrogens and, accordingly, estrogen-targeted endocrine therapies are the mainstay of neoadjuvant and adjuvant treatments. The selective ER modulator (SERM) tamoxifen has been used to treat breast cancer since the 1970s [4]. Other widely used endocrine therapies include aromatase inhibitors (AIs) (i.e., letrozole, anastrozole) that block the endogenous production of 17β-estradiol or selective ER degraders (SERDs) (i.e., fulvestrant (ICI)) that destabilize ER [5]. Some combination and duration of these endocrine agents can stabilize or eradicate luminal breast cancer in many cases. However, development of endocrine resistance, which can occur acutely or over many years, still manifests in ~40 % of patients [6, 7]. Therefore, models that recapitulate the stages of luminal tumor progression, with the capacity to address genetic diversity and tumor heterogeneity, are direly needed to improve outcome.

The plethora of in vivo research in human breast cancer has relied on implantation and growth of established cell lines into solid tumors [8]. These tumors are, however, limited in their ability to recapitulate the innate heterogeneity observed in most primary breast cancers and thus models that represent patient tumors more closely have long been sought. To this end, direct grafting of patient breast tumor material into immune-compromised mice has been attempted continuously not long after such models became available [9, 10]. Breast cancer presents a particular challenge for development of such patient-derived xenografts (PDX), as each of the described subtypes has a different prospective origin and natural course of the disease. The relatively recent emergence of more severely immune-compromised mouse strains as well as improved transplant techniques has increased the feasibility and efficiency of utilizing direct patient breast tumor engraftment. As such, the number of groups developing breast cancer PDX banks has increased in the last several years. A major interest in their development is to utilize PDX as preclinical models for testing experimental therapeutics. However, PDX are also useful for the study of tumor heterogeneity, metastasis, and signaling pathway dependencies pre- and post- drug resistance. Luminal breast cancer PDX in particular will be valuable in dissecting the role of steroid hormone receptors in the context of solid tumors.

Steroid Hormone Receptors in Breast Cancer

Steroid hormone receptors belong to the nuclear receptor family of ligand activated transcription factors. There are six defined members which contain a conserved double zinc finger DNA binding domain and are activated by lipophilic ligands that are derived from cholesterol through the process of steroidogenesis [11, 12]. ERs are encoded by two separate genes, ER-alpha (*ESR1/NR3A1*) and ER-beta (*ESR2/NR3A2*), that bind to distinct palindromic DNA sequences termed estrogen response elements (EREs). The remaining members are each encoded by a single gene and bind ligand, including progesterone receptor (PR, encoded by *PGR/NR3C3*), androgen receptor (AR, encoded by *AR/NR3C4*), glucocorticoid receptor (GR, encoded by *GCR/NR3C1*), and mineralocorticoid receptor (MR, encoded by *MCR/NR3C2*). The latter four steroid receptors (PR, AR, GR, and MR) share a similar palindromic consensus DNA binding sequence. While estrogens are the main mitogens in breast cancer, progesterone and androgens can have autonomous context-dependent actions on tumor growth [13, 14] and, through their receptors PR and AR, can directly modulate the actions of ER [15, 16]. Early clinical studies found high dose synthetic progestins and sometimes androgens were partially efficacious in treating late stage breast cancer; progestins in some studies were equivocal to tamoxifen, which thereafter became standard of care [17–23].

There is currently renewed interest in targeting both PR and AR in breast cancer. However, the divergent roles of these receptors in driving breast cancer growth complicate their use and necessitate further study for patient selection [13, 14]. There is also recent interest in the role of GR in breast cancer, particularly the ER− subtypes [24–28]. PDX models that express each of these receptors in different combinations will be critical to evaluate new steroid receptor-targeted drugs and make selections for appropriate candidates for treatments. Unfortunately, most steroid hormone receptor positive breast cancers develop into xenografts much less efficiently than their receptor negative counterparts, which usually proliferate faster and do not require hormonal stimulation.

Maintenance of appropriate steroid hormone receptor expression between patient tumors and PDX is a critical step in capturing relevant models for research and preclinical drug testing. While ER-alpha is expressed in 70–75 % of breast cancers at initial diagnosis, there is widespread heterogeneity in the level of ER expression and fidelity of ER-alpha-mediated signaling among tumors. Currently, tumors with 1 % or greater ER immunoreactive cells are recommended as candidates for endocrine therapies [29]. Furthermore, complete loss of ER-alpha upon endocrine resistance is rare; tumors usually remain clinically ER-alpha+ (≥1 %) but often bypass ER-alpha signaling and utilize receptor kinase-driven pathways such as EGFR/HER2 or PI3K/AKT/mTOR [7]. ER-beta is expressed in a significant proportion of breast cancers (76 %); however, its role in breast cancer remains elusive and ER-alpha remains the major prognostic indicator and target [30–32]. PR is expressed in over half of ER+ tumors and has been used as a functional measure of ER [33], while AR is expressed in 70–80 % of all breast cancers [34–36]. GR has been measured less frequently in breast tumors; estimates indicate between 30 and 70 % of invasive breast cancers express GR (reviewed in [37]). MR to our knowledge has not been comprehensively measured in breast cancer, although mRNA levels were detected in TN MDA-MB-231 breast cancer cells [38]. In this review, we focus on ER-alpha (termed ER), PR, and AR expression in breast PDX models.

Recent Development of Steroid Hormone Receptor Positive Breast Cancer PDX Collections

To date, at least 10 laboratories have described establishment and maintenance of steroid hormone receptor positive PDX (Table 1). Here we focus on several reports that have emerged since 2007. In the first, Marangoni et al. utilized intact female nude mice supplemented with 17β-estradiol via the drinking water [39]. In the original report, 200 primary tumors were implanted into the interscapular region which yielded 18 patient-derived lines [39]. However, only one of these was ER+ (<1 % engraftment rate compared to 36 % for TN breast

Table 1　Summary of established PDX models

Citation	Contact	Institution	Mouse strain/implantation site	Estrogen dose/route	ER+PR+ PDX	ER+PR− PDX	Total ER+ PDX	ESR1 mutants	GEO accession number
[39, 40]	Marie-France Poupon/Didier Decaudin	Institut National de la Sante et de la Recherche Medicale/Institut Curie, Hôpital St Louis (France)	Swiss nude; intrascapular	17 mg/ml, 8 μg/ml via drinking water	7	1	8	ND	N/A
[41]	Olav Engebraaten	Oslo University Hospital/University of Oslo (Norway)	SCID; dorsal flank	1.7 mg via pellet	1	0	1	ND	GSE23041
[42]	Alana Welm	University of Utah Health Sciences Center (USA)	NOD/SCID; MFP	1 mg via pellet	5	0	5	ND	GSE32532
[43]	Carol Sartorius/Peter Kabos	University of Colorado Denver Anschutz Medical Campus (USA)	NOD/SCID, NSG; #4 inguinal MFP	1 mg via silastic tubing pellet	6	6	12	D538G; amplification	GSE36075
[44]	Jane Visvader/Geoffrey Lindeman	The Walter and Eliza Hall Institute of Medical Research (Australia)	NSG; inguinal MFP	0.5 mg via pellet	13	– (not specified)	13	ND	GSE47369
[45]	Michael Lewis	Baylor College of Medicine (USA)	SCID/Bg, NSG; MFP	0.36 mg via pellet	1	2	3	ND	GSE34412
[46]	Matthew Ellis	Washington University in St. Louis (USA)	NSG; #4 inguinal MFP	1.7 mg via pellet	6	1	7	ESR1-YAP1 fusion; Amplification; Y537S; E380Q	GSE46604
[47]	Samuel Aparicio	University of British Columbia (Canada)	NSG, NRG; dorsal flank, #4 inguinal MFP	5 mg/ml subcu injections in peanut oil 2×/month	5	– (not specified)	5	ND	N/A
[48]	Carlos Caldas	University of Cambridge (UK)	NSG, NRG; dorsal flank, #4 inguinal MFP	No E2 (ovary-intact animals)	8	5 (39 not specified)	52	ND	NA
[70, 71]	Cathrin Brisken	ISREC – Swiss Institute for Cancer Research (Switzerland)	SCID/Bg, NSG; intraductal into MFP (MIND)	No E2 (ovary-intact animals)	7	3	10	ND	GSE68694, GSE74608

ND not described, *N/A* dataset reference ID not applicable, *MFP* mammary fat pad

cancer). The same group recently updated their success rate; utilizing the same methods, they have now implanted 314 ER+ tumors and developed 8 ER+ lines (a 2.5 % engraftment rate) [40]. Bergamaschi et al. implanted 26 ER+ primary tumors into NOD.Cg-Prkdc[scid] (severe combined immunodeficient—SCID) mice on the dorsal flank with 17β-estradiol pellets [41]. This yielded a single ER+PR+ PDX, and one ER−PR− PDX (which originated from an ER−PR+ tumor), a 7.7 % overall engraftment rate, and an engraftment rate of 3.8 % for ER+ PDX. The ER+ PDX resembled the original patient tumor over multiple passages by histopathologic and gene expression comparisons. In 2011, DeRose et al. described derivation of five ER+PR+ breast cancer PDX lines [42]. These originated from implantation of 49 specimens from 42 patients into female nonobese diabetic NOD.CB17-Prkdc[scid] (NOD/SCID) mice with cleared mammary fat pads and commercial slow release estrogen pellets (an overall engraftment rate of 27 % for both ER+ and ER− PDX). Three of the lines originate from serial pleural effusions of the same patient and one was also HER2+. They also described that co-injection of mesenchymal stem cells increased growth and supported tumor vascularization [42]. Estrogen dependency was maintained in the five ER+ PDX lines.

Our group, Kabos et al., utilized female NOD.Cg-Prkdc[scid-]Il2rg[tm1Wjl]/SzJ (NSG) ovary-intact mice with silastic estrogen pellet supplementation and implanted 18 ER+ specimens into the intact mammary fat pads [43]. We described establishment of five ER+ PDX lines (3 ER+PR+, 2 ER+PR−); two of these emanated from liquid metastases (one pleural effusion and one ascites), and one was HER2 amplified. All of the PDX retained estrogen dependency to some degree. To date, we have implanted 74 ER+ specimens and generated 12 transplantable ER+ PDX lines, representing a 16 % engraftment rate. In 2013, Vaillant et al. report the implanting of 108 ER+ patient tumors into the inguinal mammary fat pad of NSG mice, resulting in the development of 13 ER+ PDX lines with variable PR expression, representing a 12 % take rate [44]. These authors report that PDX tumors of subtype Luminal B, when treated with tamoxifen plus small molecule Bcl antagonists, display therapeutic response above that observed with tamoxifen alone. Zhang et al. utilized a combination of SCID/Beige and NSG mice, with estrogen pellets supplied in most cases [45]. They established a total of 32 lines from 25 patients, 3 of which are ER+; one of these PDX is ER+PR+, one is ER+PR−, and one is ER+PR−HER2+ (representing a take rate of 9.4 %). Notably, they observed that clearing the mammary fat pads and humanizing with human fibroblasts did not improve take rate regardless of patient tumor subtype (from 21.4 % overall take rate across all subtypes, down to 3.4 % take rate with humanized glands). In 2013, Li et al. reported a collection emphasizing ER+ PDX, developed in female NSG mice [46]. They implanted a total of 54 ER+ specimens, the majority of these from advanced stage disease (pleural

effusions and skin metastases) and developed 7 ER+ lines (a 13 % take rate). Notably, humanized fat pads significantly decreased the take rate of ER+ tumors (down to 3 %). They also noted that engrafted tumors have a high rate of p53 mutations (69 %). In 2015, Eirew et al. utilized NSG and NOD/Rag1^{−/−}Il2rg^{−/−} (NRG) mice to generate a collection of 15 breast cancer PDX [47]. They uniquely cultured breast cancer cells as organoids prior to implantation into mammary fat pads. For select ER+ specimens, animals received subcutaneous injections of 17β-estradiol in oil every 2 weeks. Their engraftment rate was 26 % (5/19) for ER+ tumors compared to 81 % for TN and 44 % for HER2+ER− tumors. Using single cell and deep genome sequencing, they determined that clonal selection occurs to some degree in all samples and continues through serial passaging. In 2016, Bruna et al., through collaboration with several institutions, reported the largest collection of ER+ PDX to date, in which 52 ER+ breast cancer PDX were developed in NRG and NSG mice from primary and metastatic patient tumors. Exogenous estrogens were not supplied during tumor development. Histological features, RNA expression, and copy number alterations were found to be stable through several passages. Breast cancer driver mutations were accurately represented in the PDX, although the rates of specific mutations were slightly lower than that seen in patients (i.e., PIK3CA in 27 % of ER+ PDX compared to 38–43 % of patients from METABRIC/TCGA databases).

A handful of reports utilizing PDX, in which PDX is not the primary research focus or in which PDX derivation is not described in detail, are not discussed herein. In addition, several other groups are in the process of developing breast cancer PDX banks that have not yet been reported which could increase the total number of generated ER+ lines. However, the number of established breast cancer PDX still weighs disproportionately towards the TN subtype and thus improvements are needed for capturing the wide genetic and histological variety of ER+ breast cancers, as discussed below for the mouse intraductal (MIND) method.

Methodological Considerations

Engraftment Rate of ER+ Breast Tumors

The biggest challenge to developing steroid hormone receptor positive PDX is a lower engraftment rate compared to receptor negative tumors. Estimated by several reports, this is on average less than 15 % for primary ER+ tumor samples, with potentially higher success for late stage metastases (Table 1), excluding the novel MIND method, which may yield higher success rates with difficult-to-engraft samples. This low engraftment rate is in comparison to TN breast cancers, in which primary tumors graft at 30–80 % [40, 45–47]. There are few

HER2+ER− PDX models, prospectively due to their lower patient prevalence (~15 % of all cases). Grade 3 tumors have higher engraftment rates than grade 1 or 2 tumors [45], prospectively due to a higher proliferative index. Therefore, existing ER+ breast cancer PDX favor the faster growing Luminal B subtype breast tumors. Luminal B tumors are more likely to become endocrine resistant, have a higher incidence of metastases, and have worse progression-free and overall survival than Luminal A tumors [49–51]. Luminal B tumors in general tend to have lower ER expression and increased intratumoral ER− cells which may also contribute to their better take rate. Where intrinsic molecular subtyping has been used, all ER+ PDX except one profile as Luminal B [41–43, 45, 46]. The one Luminal A PDX described by Petrillo et al. is, however, ER− [52]. Interestingly, primary tumor specimens that profile as Luminal A prior to implantation switch to Luminal B upon establishing a transplantable PDX [46]. This is likely due to selection pressure that favors relatively rapid proliferation and propagation (up to 6 months). Other histological breast tumor types such as lobular that tend to be highly ER+ are difficult to develop. Four mixed lobular/ductal breast cancer PDX have been described between two collections [42, 48]. Improved models for developing ER+ PDX, discussed below, may increase the efficiency of grafting such tumors.

The Estrogen Milieu for Establishing ER+ PDX

In most cases, establishment of ER+ breast cancer PDX requires exogenous estrogen supplementation. Multiple methods exist for in vivo delivery of estrogen. One method involves frequent (daily to weekly) injection of hormones dissolved in peanut (or similar) oil vehicle; this method, while efficacious, is labor intensive and imparts more stress on the animals. A second method is delivery via the drinking water [53]. While noninvasive, this method requires frequent preparation of fresh 17β-estradiol stocks and biweekly water changes, with some debate over bioavailability, though circulating levels induce uterine hypertrophy similarly to that observed with pellet supplementation [53]. The most common method of estrogen delivery is through slow release pellets. These are available commercially in several doses and release times from Innovative Research of America (Sarasota, FL) and can be inserted subcutaneously via trochar. Alternatively, some laboratories prepare their pellets in-house using commercially available hormone stocks; the most common method involves packaging hormones within silastic medical tubing [54]. With this method, the dose of hormones can be manipulated, is suitable for use with steroid hormones (17β-estradiol, progesterone, dihydrotestosterone), and lasts for 3–4 months. Circulating levels of steroid hormones can be monitored relative to dose using ELISAs or RIAs; 17β-estradiol is in the high physiological range, estimated at

<100 pg/mL for silastic pellets (1 mg) [55]. By survey, most groups use intact animals for establishment of PDX with chronic estrogen supplementation. For experiments beyond initial passaging, animals may be ovariectomized to provide the cleanest hormone negative background where necessary.

One of the first routine experiments conducted upon successful engraftment of an ER+ tumor specimen is evaluation of its dependency on estrogen for growth. In our hands, tumors are partitioned into animals upon passage 1 or 2 that are supplemented with placebo or slow release silastic estrogen pellets to document the degree of estrogen dependency. In our experience, some highly ER+ tumors will not form tumors in ovary-intact mice without exogenous estrogen; there is little to no residual material at necropsy 3–4 months after implantation. Other PDX show partial growth in the absence of exogenous estrogen; these are usually late stage tumors or primary tumors with lower ER expression. In addition, we often monitor relative tumor growth in the presence of estrogen plus either progesterone or the androgen dihydrotestosterone. The tumors we have tested do not grow significantly larger than placebo with either progestins or androgens alone.

Maintaining Steroid Receptor Expression and Function in PDX

Another major hurdle in development of steroid receptor positive PDX is loss of receptors upon establishment and/or propagation. Ideally, receptor expression is initially monitored through routine parallel immunostaining comparing the PDX to the original tumor specimen. However, this is not always feasible due to limiting material and/or availability, or staining challenges inherent with fluid samples. From our own experience and from other reports, patient ER levels are initially captured in the majority of established ER+ PDX. Careful evaluation of ER staining at each passage is then necessary, and if lost, that PDX should be reevaluated for usefulness as a model. ER undergoes ligand-dependent down-regulation and this should be considered when evaluating ER expression in breast cancer PDX [56–59]. The constant low dose estrogen necessary to propagate ER+ tumors will also yield chronic cycling of the transcriptionally active ERs. Removal of estrogen for several weeks (estrogen withdrawal, EWD) can lead to an increase in ER levels (Fig. 1a). Similarly, tamoxifen treatment can stabilize ER protein and lead to an observed upregulation of ER in PDX. In our experience, PDX that appear to have low or absent ER levels will show an increase in detectable ER upon tamoxifen treatment, signifying a low but active level of ER (Fig. 1b). This is important as it mimics clinical tumors with lower ER that may be less dependent on estrogens and are clinically difficult to treat. Conversely, SERDs such as ICI will decrease ER levels in breast cancer (Fig. 1b). Therefore, it is always important to consider the hormonal context under which ER is measured in breast cancer PDX.
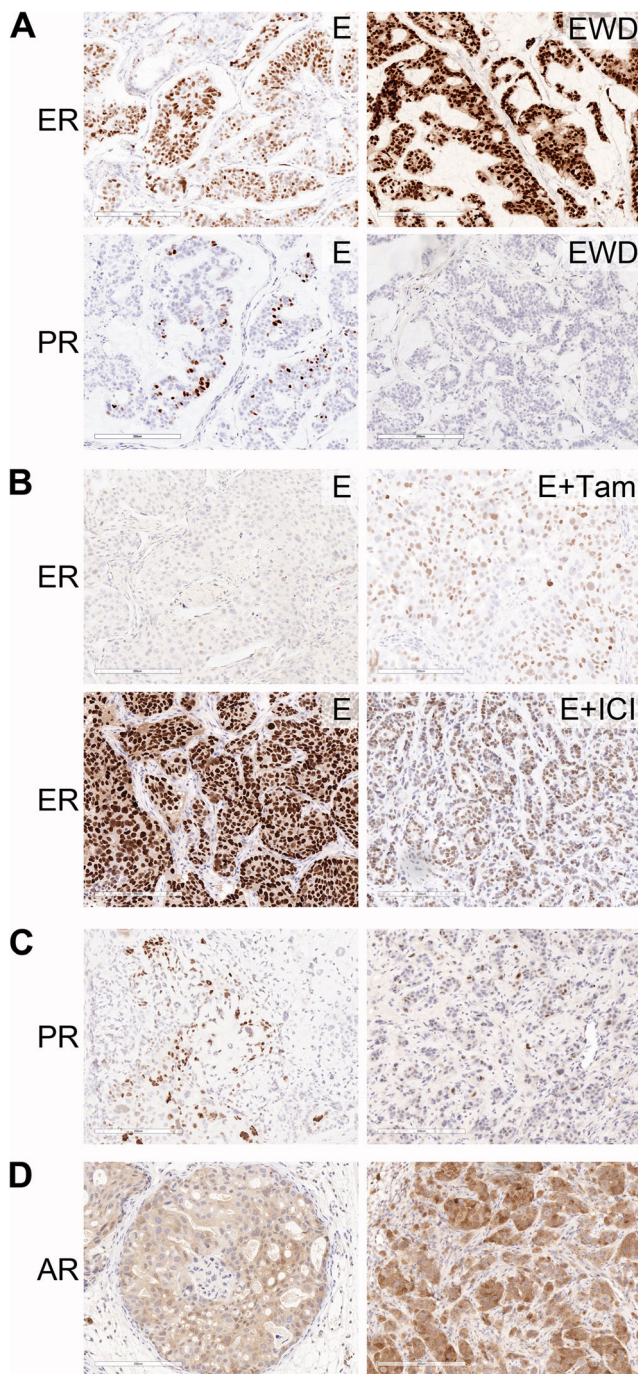
Fig. 1 Context-dependent expression levels of ER, PR, and AR in breast cancer PDX. Formalin-fixed paraffin-embedded sections of select PDX breast tumors were processed for immunohistochemistry as previously described [43]. Tumors were stained with antibodies to ERα (Thermo-Fisher, clone SP1, 1:100), PR (DAKO, clone 1294, 1:500), or AR (DAKO, clone AR441, 1:500). **a** ER and PR expression fluctuate with estrogen levels. Sections of PDX UCD12 were stained for ER and PR from estrogen-treated tumors (*E*, silastic pellet) or from tumors that were withdrawn from estrogen for 3 weeks (*EWD*). **b** ER expression fluctuates with endocrine treatments. Sections of UCD4 were stained by IHC for ER under conditions of estrogen plus either vehicle (*E*) or tamoxifen (*Tam*, administered as 1 mg Tam dissolved in peanut oil IP 3× per week for 3 weeks), or under conditions of estrogen plus vehicle or ICI (5 mg ICI in peanut oil IP 1× per week for 5 weeks). **c** PR expression in two different TN breast cancer PDX (UCD18 and UCD111). **d** AR expression in HER2+ER− tumor UCD62 and TN tumor UCD138. *Scale bars* 300 μm

(Fig. 1a). In our collection, 6/12 ER+ tumor lines retain PR expression (one undetermined), reflective of its clinical prevalence. However, in two of these, expression levels are <10 %, making it difficult to study PR action. ER+PR− PDX breast cancers have poorer prognosis overall than their ER+PR+ counterparts [60], and thus these models will be useful for studying this subset of the disease. We have noted that patient tumors that are characterized as TN occasionally generate PDX that express PR in <10 % of cells, in the absence of ER or supplemented estrogens (Fig. 1c).

AR is present in 70–80 % of all breast tumors, with varying expression across the different subtypes. AR is found in approximately 80 % of ER+ tumors, over half of HER2+ER− tumors, and 10 % of TN breast tumors [34–36, 61, 62]. At present, AR is not routinely measured in clinical samples. Therefore, retention of AR expression in PDX needs to be compared to the primary patient specimen by parallel immunohistochemistry (IHC) where feasible. In our collection, AR is expressed in >1 % of cells in 7/11 ER+ PDX (one undetermined). Expression levels are highly variable similar to ER and PR; some lines contain very low AR (<5 %), making them less than ideal for most studies. Our one HER2+ER− PDX line is AR+ and, where measured, 2/6 TN PDX lines are AR+ (Fig. 1d). Each of these types of PDX models will be valuable as AR is being investigated as a putative target in ER+ and ER− tumor subtypes [63–65]. Unlike ER and PR which reside predominantly in the nucleus in both the absence and presence of ligand, AR is usually cytoplasmic and is shuttled to the nucleus with addition of an androgenic ligand. This is recapitulated in breast cancer PDX.

In contrast to most breast cancer cell lines, steroid receptor positive PDX generally retain the inherent intratumoral heterogeneity associated with patient tumors. For ER in particular, this includes a range of intensities and levels of receptors, ranging from <5 to near 100 % positive cells. This natural mosaic of ER expression underscores the challenge of treating ER+ tumors. It also may allow for the study of mixed populations, for example, ER+ and ER− cells within the same

PR at present is the only other steroid receptor routinely measured in breast cancer. PR is present in over half of ER+ tumors [33]. It has been recently described that the PR locus is subject to copy number loss, which may account for its overall lower abundance relative to ER in breast cancers [15]. In addition, PR expression is particularly difficult to retain in PDX. PR expression in breast cancer is usually estrogen dependent. Thus, the chronic estrogen required to establish and propagate ER+ PDX should assist in maintaining PR expression where possible; likewise, EWD can lead to loss of PR expression

tumor microenvironment. However, heterogeneity can impede the use of molecular technology to study steroid receptor action. Gene regulation, for example, can be washed out by the prevalence of receptor negative cells. Furthermore, techniques such as chromatin immunoprecipitation followed by sequencing (ChIP-seq) and rapid immunoprecipitation followed by mass spectrometry of endogenous proteins (RIME) [66] for steroid receptors are not currently feasible on many PDX tumors, which require high concentrations of receptors for sufficient immunoprecipitations. Primary patient specimens are often too limited in size for these techniques such that expansion as PDX is attractive for such studies in the event that high receptor levels are maintained.

Newer Techniques That Improve Engraftment Rates of ER+ Tumors

Several adaptations to implantation methods have been tested to improve take rate, including choice of mouse strain, use of cleared mammary fat pads plus/minus humanization of the mammary gland, and co-injection of mesenchymal cells. Nude mice (NU/J) have the highest remaining murine immunity (retain B cells) and thus display the lowest engraftment efficiency for ER+ tumors (2.5 %) [40], and therefore most groups have moved to strains with more severely immune-compromised systems such as SCID/Bg (CB17.Cg-Prkdc^{scid}Lystbg-J/Crl), NOD/SCID, and NSG. Once PDX are established in these mice, it is feasible to backtransplant them to other strains. For example, TN PDX established in NSG mice can be backtransplanted efficiently to nude mice [67]. We have also backtransplanted ER+ PDX from NSG to B6-RAG1 (B6.129S7-Rag1^{tm1Mom}/J) mice successfully with no observable loss of receptor expression or tumor phenotype. Humanizing the mammary gland does not improve and may in fact decrease take rates [45, 46], while co-injection of human mesenchymal stem cells can accelerate tumor vascularization and growth [42, 68].

An implantation technique termed MIND (mouse intraductal) was developed by Behbod et al. for in vivo study of ductal carcinoma in situ (DCIS) [69]. In this method, cells are injected directly into the milk ducts and form structures within the mammary fat pad. This technique was adapted by Sflomos et al. for breast cancer cell lines and primary tumor specimens using ovary-intact (low estrogen) SCID/Bg or NSG mice [70, 71]. MCF7 cells implanted into the fat pads using traditional techniques displayed a higher expression of basal and epithelial-mesenchymal transition (EMT) genes including SLUG compared to the MIND method of implantation. Furthermore the MIND-implanted tumor cells had higher ER expression levels, suggesting that the MIND method maintains a more luminal-like phenotype in tumors.

To establish MIND PDX, primary patient tumors were dissociated into single cell suspensions and 5000–10,000 cells injected into the ducts. Engrafted cells retained the original ER and PR expression levels, and resulted in the generation of seven ER+PR+ and three ER+PR− PDX lines (Table 1). MIND engraftment rates with ER+ primary patient samples were higher (17–100 % take rate, depending on patient sample) than is generally observed for intrascapular or mammary fat pad implantations of ER+ patient samples (2.5–27 %, from multiple collections summarized in Table 1) [71]. Importantly, lower grade tumors (1 and 2), Luminal A tumors, and lobular tumors, subtypes that historically have proven difficult to engraft, all established growths with the MIND method, and PDX tumors maintained both histopathological similarity and proliferative indices as primary tumors. Growth kinetics of luminal-subtype tumors are frequently altered while establishing PDX due to selection pressure, as a clonal population of fast-growing cells may not reflect the growth kinetics of the original tumor; hence, the predominance of Luminal B PDX. Thus, the MIND approach could allow for a more robust representation of the spectrum of ER+ tumors. One disadvantage is that the quantity of material may be limiting for larger scale experiments (i.e., dispersal into multiple animals for preclinical drug testing or proteomics-based experiments).

Caveats and Limitations of ER+ Breast Cancer PDX

There are several caveats inherent to all PDX that also apply to steroid hormone receptor positive breast cancer PDX. These include acquisition of proliferating murine stroma, development of transferable murine malignancies, viral contamination, and potential genetic drift over passaging. Murine stroma that propagates with whole fragments of tumors can become activated and spontaneously immortalized such that it proliferates faster than steroid hormone receptor positive breast cancer epithelial cells. In such cases, the murine cells take over the tumor within one to two passages. Loss of hormone receptor expression, loss of human cytokeratin expression, and gain of a squamous spindly appearance all indicate a problem. If this occurs, it may be possible to reconstitute the line from earlier passages, but research on the current specimen should be discontinued.

Use of immunocompromised mice brings additional considerations. NOD/SCID animals in particular are susceptible to lymphoma [72]. This can passage with the PDX leading to rapid growth of solid masses of murine cells and necessary euthanasia of the animals prior to PDX regrowth. In contrast, NSG mice are less susceptible to lymphoma. In our experience, while we have had occasional development of lymphoma using NOD/SCID mice for long incubation periods (~6 months), we have not observed this phenomenon in NSG mice. Viral contamination is another recurring issue that may arise while propagating tumors in mice [73]. The most problematic viral contamination is lactate dehydrogenase elevating virus (LDEV). This is a common murine viral

contaminant that can be found in commercially available basement membrane extract (BME) preparations that are frequently used when implanting tumor cell suspensions or solid tumor pieces [73]. The virus can take several passages before physical signs appear, at which time animals develop partial or full paralysis. The virus is transferred via direct tissue/fluids and infects murine macrophages [74]; plasma, liver, and spleen tissue from infected mice display the highest LDEV titer [75].

Two methods have been described to eliminate LDEV. The first includes FACS to eliminate all murine cells from a single cell preparation, followed by reimplantation of human tumor cells into new animals [76]. However, this requires serial sorting, and unless the sort is near 100 % efficient, the virus will still transfer. A second, older method, albeit one still recommended by Charles River, involves passage through nude rats which eliminate the murine macrophages. The sample can then be evaluated for loss of LDEV via qPCR and reimplanted into mice. The tumor would likewise need to be monitored for appreciable morphological changes. While this approach may be feasible for TN tumors, in our experience the continuous estrogen supplementation required to maintain ER+ tumor samples can lead to the appearance of spontaneous rat tumors that interfere with the slower growing human ER+ PDX. Most present sources of BME are LDEV-free which has mostly eliminated this problem (i.e., Cultrex®, Trevigen, Gaithersburg, MD).

The stability of the breast cancer PDX phenotype over passage is an important consideration and potential caveat that has not been entirely resolved in the field. Eirew et al. described that the major selection of patient-derived breast cancer cells fit for propagation as a PDX occurs in many cases during initial establishment, although some drift occurs through every subsequent passage [47]. Where measured, some PDX collections appear to retain copy number variations and SNPs compared to the primary patient sample over multiple passages, with occasional gain in activating SNPs [41, 42, 45]. Gene expression measured via Affymetrix analysis and proteome analysis via RPPA were found to be relatively stable measured every fifth passage [45]. Bruna et al. extensively monitored genomic and molecular fidelity using single nucleotide variations (SNV), copy number alterations, gene methylation, and gene expression profiling, and concluded that PDX tumors maintain high correlation with patient tumors across serial passages [48]. Deep sequencing identified single nucleotide variations accumulate out to passage 8 in ER+ PDX and were predominantly in non-cancer associated genes [46], a conclusion recently confirmed by Bruna et al. [48]. In the MIND model, PDX tumors did not accumulate de novo mutations over passage and thus may be more genetically stable [71].

While no consensus passage number exists at which PDX are reliable to utilize, commercially contracted PDX studies usually restrict the use of their models to within a few passages removed from the original patient sample. However, this is not always practical for steroid receptor positive breast cancer PDX which often grow slower and can take a few passages to establish and expand prior to performing experiments. Therefore, for preclinical studies on developmental therapeutics, the lowest possible passage should be used. Careful replenishing of frozen stocks and reimplantation of lower passages can prolong experimental use with the caveat that lower passage stocks will eventually be depleted. With diligent monitoring via IHC and other methods capable of detecting gene expression changes, ER+ PDX can be reasonably used for four to five passages for many types of studies. To minimize the usage of limited stock, Bruna et al. recently reported an ex vivo culture method wherein cell lines derived from PDX breast cancer tumors could be used for high throughput screening of single agents and combination therapy [48], which allows for pre-validation of therapeutic agents to minimize inefficient use of limited PDX stock. It is unknown at this time if the precise method of breast cancer PDX establishment and maintenance influences therapeutic response.

Steroid Hormone Receptor Positive PDX: New Avenues for Investigation

Gene Expression Profiling of Steroid Hormone Receptor Positive PDX

Steroid hormone receptor positive PDX offer new opportunities for defining hormone-dependent gene regulation. ER and PR regulated genes in breast cancer have been mainly defined by the immortalized cell lines MCF7 and T47D, respectively [77–81]. In addition, several clinical trials utilizing endocrine therapies have generated datasets reporting estrogen-dependent genes that are deposited in publically available databases [82–84]. Our group evaluated gene expression in PDX plus/minus estrogen, tamoxifen, and EWD [43]. In comparing three ER+ PDX to MCF7 cells, there were many similar estrogen-regulated genes such as *GREB1*, *IGF1R*, and *PGR*. Not surprisingly, each ER+ PDX contains tumor-unique estrogen-regulated genes. For example, in PDX UCD4 (PE4 in the original manuscript), estrogen highly up-regulated a group of genes located on the X chromosome termed cancer testis antigens (*CT45*), occasionally observed in multiple tumor types that are candidates for immunotherapies [85]. In our experience, progestin regulated genes are more variable among PDX. A preliminary comparison of overlap between PR regulated genes in PDX compared to those described in T47D cells noted only a few consistently PR regulated genes (i.e., *TAT* and *CXCL13*) (unpublished data). This coincides with the emerging functional heterogeneity that PR appears to convey in ER+ breast cancers. Assessment of PR regulated

genes may pinpoint those tumors in which PR acts as an ER agonist vs. antagonist.

Endocrine Resistance and ER Mutations

There are limited models to study endocrine resistance in ER+ breast cancers. The most commonly used models are tamoxifen-resistant MCF7 breast cancer cell lines, which have proved valuable in investigating mechanisms of tamoxifen insensitivity [86, 87]. To generate similar endocrine-resistant PDX, samples must be implanted from pre-existing resistant tumors, or resistance manifested utilizing a chronic in vivo treatment protocol similar to that utilized for MCF7 cells in vitro. This strategy was described for a primary breast tumor implanted in nude mice over 3 years of tamoxifen treatment [88]. The Washington University collection contains ER+ PDX lines that were developed from late stage cancers that were endocrine resistant [46]. Interestingly, four of their ER+ PDX lines contain ER variants: an *ESR1/YAP1* fusion protein resulting from a translocation event (WHIM18); amplification of the *ESR1* promoter and coding region (WHIM16); an *ESR1-E380Q* point mutation in the ligand binding domain (WHIM24); and an *ESR1-Y537S* point mutation in the ligand binding domain (WHIM20) [46]. In our collection, UCD4, developed from a pleural effusion, harbors the *ESR1-D538G* mutation, and UCD65 displays *ESR1* amplification. ER mutations are emerging as more prevalent than originally thought, likely due to improved sequencing and detection technologies [89, 90]. Naturally occurring ER mutant PDX models will be useful for testing novel ER mutant targeting drugs under development, particularly newer generation SERDs. In addition, companion cell lines could be generated from such tumors, albeit with no mutation negative control. These types of models will compliment CRISPR/Cas9-generated ER mutants in breast cancer cell lines. Mutations in PR and AR have not yet been described in breast cancer, likely since mutations are primarily driven by pressuring the ER signaling axis.

One inherent limitation of breast cancer PDX is the lack of an intact immune system. Immune cells and associated inflammation affect multiple components of tumor biology. Humanized models are being developed in which irradiated mice are reconstituted with human cells, including T cells, myeloid cells, and hematopoietic progenitor/stem cells, resulting in mice regaining partial immunity [91–93]. As the technology becomes more refined and available, PDX may be established and/or transplanted into animals with human-like immune systems. This may facilitate processes such as metastasis, which occur but are inefficient in immune-compromised hosts.

Conclusions

The recent upsurge in the development of breast cancer PDX has naturally led to generation of ER+ PDX; at least 116 transplantable lines exist worldwide. Many of the ER+ PDX co-express PR and AR, and several are also HER2+/amplified. While the process of ER+ PDX derivation is still relatively inefficient (<20 %), as technologies improve, these collections may allow for a more robust representation of ER+ breast cancers. In particular, utilization of the MIND approach may capture tumor types not amenable to solid specimen transplant into mammary fat pads. Breast cancer PDX retain the inherent heterogeneity in ER, PR, and AR expression, making them attractive for preclinical studies aimed at testing drug efficacy in a spectrum of the disease. Furthermore, primary tumor-derived PDX that were untreated or undertreated offer an advantage for testing new endocrine agents. In addition to anti-estrogen-targeted drugs, these may help delineate tumors that would respond to therapies with natural steroid hormones such as progesterone and androgens vs. those that could be stimulated by such treatments. In conclusion, steroid hormone receptor positive breast cancer PDX allow an unprecedented opportunity to study endocrine resistance and steroid receptor action in solid tumor models that more closely represent human breast cancer biology.

Compliance with Ethical Standards

Conflict of Interest   The authors declare that they have no conflict of interest.

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