Modeling Breast Cancer Intertumor and Intratumor Heterogeneity Using Xenografts

ALEJANDRA BRUNA,¹ OSCAR M. RUEDA,¹ AND CARLOS CALDAS^{1,2}

¹*Department of Oncology and Cancer Research UK Cambridge Institute, Li Ka Shing Centre, University of Cambridge, Cambridge CB2 0RE, United Kingdom*

²*Breast Cancer Programme, Cambridge Cancer Centre, Cambridge CB2 2QQ, United Kingdom*

Correspondence: carlos.caldas@cruk.cam.ac.uk

Breast cancer is a heterogeneous disease that can be stratified in at least 10 different subtypes. We present here a platform for derivation of preclinical models based on patient-derived tumor xenografts (PDXs) that represent these subgroups. These models preserve the transcriptome, methylome, copy-number, and mutational landscape features of the tumor of origin through different passaging. Furthermore, the intratumoral composition of these models is formed by communities of clones very similar to the ones present in the originating tumor. Finally, we show that short-term cultures of cells from these models (PDX-derived tumor cells, PDTCs) also preserve the molecular features of the tumor and can be used for high-throughput drug testing of single compounds or combinations, with high reproducibility and clinical predictive power.

Breast cancer is a heterogeneous disease. Consequently, precise breast cancer stratification is a crucial step toward more efficacious treatment. For many years patients have been stratified based on clinical–pathological features including staging, tumor grade, and the estrogen receptor (ER), progesterone receptor (PR), and Her2 status. Molecular stratification based on gene expression profiling defined the five intrinsic subtypes of breast cancer: basal-like, Her2, luminal A, luminal B, and normal-like (Perou et al. 2000).

However, these classifications are not optimal. Clinical and histological stratification still fails to assign patients to the right treatment; in particular within the largest subset of patients, which are ER⁺/Her2[−], where hormone therapy, with Tamoxifen or aromatase inhibitors, frequently fails, and where use of chemotherapy is fraught with poor prediction of benefit. This problem persists even using the gene expression–based intrinsic subtype classification, where luminal A and luminal B are very broad groups that only differ in proliferation status. We therefore hypothesized that a taxonomy of breast cancer based on genomic driver events would be an improvement, which would ultimately lead to better treatment decisions. The METABRIC (Molecular Taxonomy of Breast Cancer International Consortium) project (Curtis et al. 2012) used copy-number and expression profiling of 997 primary breast cancer samples to identify the top 1000 candidate driver genes based on their expression being driven in *cis* by copy-number aberrations. Using these 1000 *cis*-driven genes, enriching for oncogenes and tumor-suppressor genes, we showed that breast cancers could be divided into 10 integrative clusters (IntClusters). This molecular stratification was validated in a further 983 samples from the same cohort (Curtis et al. 2012) and subsequently

in several external data sets (Ali et al. 2014). Some of the IntClusters were characterized by a very clear copy-number driver event, like the *CCND1/PAK1* amplification in IntCluster 2, the *HER2* amplification in IntCluster 5, or the *MYC* amplicon in IntCluster 9, but in some other groups drivers were more elusive. To gain insight into different molecular events, we also performed targeted sequencing of 173 genes (Ali et al. 2014) that are relevant in breast cancer, finding that 40 of them are drivers (mut-driver genes), with different frequencies in each IntCluster. This new layer of molecular data contributed to refining the molecular landscape of breast cancer. Significantly, some of these mutation drivers seem to have IntCluster specific prognostic value. For example, *PIK3CA* is associated with worse survival in only three ER⁺/Her2[−] subgroups. We also quantified the level of intratumoral heterogeneity in individual tumors, revealing its distribution is distinctly different between IntClusters. Importantly we also showed that intratumoral heterogeneity is a biomarker of disease-specific survival.

However, all of these important biological associations that we can infer with a well-annotated and well-characterized cohort like METABRIC need a set of representative preclinical models in order to be validated. With this aim in mind, we developed a classifier to assign existing breast cancer cell lines into one of the IntClusters (Curtis et al. 2012). We observed the fit was poor because the genomic profiles of the cell lines did not reflect well those found in breast cancer samples in the clinic. This is a consequence of the nature of cell lines, which not only fail to capture the intratumor and intertumor heterogeneity seen in the clinic but also have other limitations probably reflecting adaptation to long-term culture in plastic. These drawbacks are thought to have strongly impacted

cancer drug development, which has suffered from high attrition rates, mainly because of lack of efficacy in the clinic (Harrison 2016).

DEVELOPING A COLLECTION OF PATIENT-DERIVED TUMOR XENOGRAPHS

Patient-derived tumor xenografts (PDXs) have emerged as tools with the potential to improve cancer drug development (Tentler et al. 2012; Hidalgo et al. 2014; Aparicio et al. 2015; Gao et al. 2015). We therefore set out to develop PDXs as a better model to study drug sensitivity but also to reflect tumor evolution under the selective pressure produced by treatment, enabling the study of mechanisms of sensitivity and resistance (Fig. 1). We have now optimized a framework to efficiently engraft breast cancer samples from the hospital into immunocompromised mice (NSG). With time we have substantially improved this platform to (1) ensure rapid and efficient implantation of new breast cancer samples, (2) expand the successful engrafted samples through serial transplants for biobanking purposes, and (3) provide these models as a research tool. The breast cancer PDX biobank is now one of the largest in the academic setting and is composed by almost equal number of ER⁺ and ER⁻ samples, most of which are from breast tumor primaries.

We have now robustly shown that the PDXs represent the breast cancer patient population and preserve most of the features of the originating tumor sample. This has included full characterization at the transcriptome, methylome, copy-number, and mutational landscape levels, demonstrating similarity with the originating human tumor material and its preservation through several passages in the mouse.

We soon realized the downstream analysis of sequencing data was complicated by the nature of PDX samples, which are unknown mixtures of mouse stromal and hu-

man epithelial malignant cells. This prompted the development of a bioinformatics pipeline that allowed for the identification with 99.9% accuracy of mouse and human DNA sequences. Significantly, we have observed the proportion of mouse stromal cells in the PDXs remains stable through serial passages. These data were confirmed by orthogonal methods such as fluorescence in situ hybridization (FISH) and immunohistochemistry (Bruna et al. 2016).

We used exome sequencing to define single-nucleotide variations (SNVs) and small insertions and deletions (indels), shallow whole-genome sequencing to obtain copy-number profiles, Illumina expression microarrays (and RNA-seq) to measure gene expression and infer pathway activation, and reduced representation bisulfite sequencing (RRBS) to characterize DNA methylation.

We classified PDXs into the IntClusts using the method we previously described (Ali et al. 2014). The copy-number profiles of PDXs classified into each IntClust are similar to those reported in primary tumors, contrasting to what we observed in cell lines (Curtis et al. 2012; Ali et al. 2014). The subtype distribution of engrafted PDX samples in the biobank is biased toward ER⁻ and ER⁺ tumors with worse prognosis (Curtis et al. 2012; Bruna et al. 2016), reflecting the favored engraftment of more aggressive breast cancer subtypes. Hence, the ER⁻ PDX samples in the biobank have a distribution of most frequently mutated genes similar to that found in ER⁻ breast cancers from the The Cancer Genome Atlas (TCGA) cohort (Cancer Genome Atlas 2012). In contrast, frequencies of mutated genes in ER⁺ PDX samples mirrored those found in more aggressive ER⁺ subtypes (Eirew et al. 2015).

When comparing PDX to their matched originating sample and to later passages, we observed that overall the molecular profiles are remarkably similar. The biggest drift occurs with engrafting, although it is model-specific, suggesting there is not a common mechanism underlying changes observed when implanting. It is hence more likely that patient- and tumor-specific determinants, in combination with growing in a new environment in the immunodeficient mouse host, affects the observed drifts. Significantly, all the molecular traits examined remained remarkably stable through serial passages in the mouse, even after more than 10 passages (Bruna et al. 2016).

We further investigated the intratumoral composition and architecture of PDX samples and observed xenografts are communities of clones with varying degrees of complexity similar to those observed in the breast cancer clinical population, and these communities share most of the clones found in the matched originating cancer sample (Bruna et al. 2016; Pereira et al. 2016). Because cancer genomes constantly evolve under selective pressure, we investigated the extent of the human-to-mouse and mouse-to-mouse clonal dynamics using PyClone (Roth et al. 2014; Eirew et al. 2015), a method for estimating the proportion of cells in a tumor carrying each mutation and clustering them into subclones (groups of cells with the same genotype). We observed that most of the clones remained stable after engraftment and throughout passag-

Figure 1. Modeling breast cancer intertumor and intratumor heterogeneity. PDXs, patient-derived tumor xenografts.

Figure 2. Patient-derived tumor xenografts (PDXs) retain most of the molecular features of their originating tumor samples, including the intratumor heterogeneity, and remain very stable in the mouse. (Portions adapted from Bruna et al. 2016.)

ing, although in certain instances some subclones increased or decreased their prevalence or even appeared or disappeared. In fact, only 20% of the almost 200 clonal clusters detected changed significantly. Importantly, only four of these contained known breast cancer drivers. We further investigated the temporal and spatial intratumoral heterogeneity in a few examples. By comparing the clonal cluster composition and allele frequency distributions in matched primary, metastatic, and PDX samples we observed both common and private clones. We further engrafted five spatially separated biopsies from a patient tumor sample and observed minor spatial intratumor heterogeneity. However, clonal dynamics were remarkably similar in independently engrafted mice regardless of their prevalence in the originating human tumor sample. Altogether, these results significantly added to previous data showing clones follow deterministic evolutionary trajectories (Fig. 2; Eirew et al. 2015).

The potential value of PDXs gained further momentum with a study by Novartis showing xenografts captured the interpatient drug response variability (Gao et al. 2015). This suggested PDXs would reach their full potential if their use would be possible for high-content studies. However, it is not feasible to do this in mice for ethical and economic reasons. This led us to develop a pipeline that would maintain and expand cancer samples *in vivo* in mice and then use the xenografts to generate single cells for short-term culture and high-throughput drug screening. These short-term cultures from PDXs (PDX-derived tumor cells or PDTCs) retain similar molecular features, including the intratumor heterogeneity, as in the originating PDX sample (Fig. 3; Bruna et al. 2016). We then did a proof of concept screen, using the Wellcome Trust Sanger Institute setup for high-throughput drug testing in cell lines (Garnett et al. 2012), in 22 PDX

models at different passages. We tested 102 compounds relevant to cancer, either as single agents or as drug:drug combinations in a 5×5 matrix of standard of care agents cisplatin and paclitaxel with six clinically relevant targeted compounds. We observed the technical and biological reproducibility was similar to that reported in established cell lines and organoids (Garnett et al. 2012; van de Wetering et al. 2015). To further verify the robustness of the data, we tested eight PDTC models using in parallel three end point DNA content- or ATP-dependent assays and observed highly correlated drug responses independently of the assay used. We also observed a very high correlation of responses in all models tested to pairs of compounds targeting the same pathway or sharing a similar mechanism of action. Furthermore, as an example of successful drug:drug combination approaches, we validated synergy between an HSP90 inhibitor and paclitaxel in Her2⁺ models (Bruna et al. 2016). These results support the idea that the PDX/PDTC platform generates biologically robust data and shows its potential of becoming a resourceful intermediate in drug discovery. To further support the value of the PDX/PDTC platform as an improved preclinical tool with clinical predictive power, we validated a set of *ex vivo* drug responses *in vivo*. Out of the 40 selected *ex vivo* drug responses, 33 were validated *in vivo*, even though different compounds with similar specificity had to be used in some cases.

In summary, we have redefined the molecular taxonomy of breast cancer using a genomic-driver-based stratification, and we have now generated a set of xenografts that model the intertumor and intratumor heterogeneity seen in the clinic. We have demonstrated these models can be used as a robust preclinical drug testing platform, and we will use them also for other perturbations to test functional heterogeneity.

Figure 3. Generation of short-term cultures of PDTX-derived tumor cells (PDTCs) that preserve the proportion of mouse and human cells, the molecular features, and the intratumor heterogeneity of its originating sample. (Portions adapted from Bruna et al. 2016.)

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