Precision modeling of breast cancer in the CRISPR era

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Genetic dissection of cancer development, therapy response and resistance in mouse models of breast cancer

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Abstract

The cancer genomics revolution has rapidly expanded the inventory of somatic mutations characterizing human malignancies, highlighting a previously underappreciated extent of molecular variability between and within patients. Also in breast cancer, the most commonly diagnosed malignancy in women, this heterogeneity complicates the understanding of the stepwise sequence of pathogenic events and the design of effective and long-lasting target therapies. To disentangle this complexity and pinpoint which molecular perturbations are crucial to hijack the cellular machinery and lead to tumorigenesis and drug resistance, functional studies are needed in model systems that faithfully and comprehensively recapitulate all the salient aspects of their cognate human counterparts. Mouse models of breast cancer have been instrumental for the study of tumor initiation and drug response, but also involve cost and time limitations that represent serious bottlenecks in translational research. To keep pace with the overwhelming amount of hypotheses that warrant *in vivo* testing, continuous refinement of current breast cancer models and implementation of new technologies is crucial. In this review, we summarize the current state-of-the-art in modeling human breast cancer in mice, and we put forward our vision on future developments.

Breast cancer is the most commonly diagnosed invasive cancer worldwide, with more than 1,6 million new cases each year. Rather than a single disease, it represents a spectrum of malignancies, encompassing several distinct biological entities and subtypes, each associated with specific histopathological and molecular characteristics, responses to therapy and clinical outcomes. Multiple taxonomies have been developed to divide breast cancer cases into different categories. Histopathological classification comprises several morphological and immunohistochemical phenotypes that can be further divided into different grades. Among the various morphologies, advanced mammary tumors mostly fall into the class of invasive ductal carcinomas (IDC), followed by invasive lobular carcinomas (ILC). Molecular classification based on gene expression patterns distinguishes five major subtypes of breast cancer: luminal A and B, ErbB2^{+}, basal-like and claudin-low (Perou *et al.,* 2000). Although these distinctions have proven useful for clinical decision-making, there are limitations in predicting disease prognosis and response to therapy. For example, a recent prospective, randomized phase-III study showed that nearly half of the women with early breast cancer who are at high risk based on standard clinicopathological parameters, might not require adjuvant chemotherapy (Cardozo *et al.*, 2016). The additional use of a 70-gene expression signature may help to identify breast cancer patients who do not require adjuvant chemotherapy, but the identification of molecular signatures that reliably predict chemotherapy response remains elusive. Moreover, sequencing studies have shown that even within the same molecular subtype an extreme heterogeneity in the mutational landscape exists, which may account for discrepancies in prognosis and therapy response between different patients (Cancer Genome Atlas Network, 2012; Stephens *et al.,* 2012; Nik-Zainal *et al.,* 2016). Another complicating factor is intratumoral heterogeneity. Individual tumors are mosaics of multiple clones of neoplastic cells, each characterized by a distinct genetic makeup and differential responses to the selective pressures to which they are exposed, making the tumor mass not static but continuously shaped by a branching evolutionary process resembling Darwinian evolution. Distinguishing causal disease variants (driver mutations) from background alterations (passenger mutations) is a major goal in breast cancer research, as it can pinpoint evolutionary conserved processes that mammary tumor cells apply during step-wise transformation and to which they might be addicted. To exploit these potential Achilles' heels, we require a comprehensive knowledge of how these signaling networks physiologically function, how they become aberrant and how they can be directly or indirectly disrupted.

Given this complexity, genetically engineered mouse models (GEMMs) of breast cancer, together with patient-derived tumor xenografts (PDX) and GEMM-derived tumor allografts, have proven valuable resources for deepening our understanding of how mammary tumors initiate, progress, metastasize and respond to therapy in a physiologically relevant *in vivo* setting (Vargo-Gogola and Rosen, 2007). These mouse

models are increasingly being used in longitudinal preclinical studies for translation of novel therapies to clinical testing. Moreover, GEMMs provide unique opportunities to infer cause-effect relationships on *de novo* induced malignancies growing in intact organisms, rather than correlative observations on end-stage patient tumor samples.

Over the past 15 years, our research has been focused on the generation and characterization of mouse models for two breast cancer subtypes: invasive lobular carcinomas and basal-like invasive ductal carcinomas. To achieve this, we engineered a number of tumor-specific driver mutations in the relevant target cells of mouse models, recapitulating the key dependencies of the resulting lesions to the corresponding deranged signaling pathways. In this review, we will discuss how these models can be used for functional dissection of tumorigenic cascades, unraveling new therapeutic vulnerabilities and mechanisms of therapy resistance; in particular in light of the advent of new technologies such as CRISPR-Cas9 gene editing, which are opening new avenues in breast cancer modeling in mice.

Invasive lobular breast carcinoma (ILC) models

ILC accounts for 8-14% of all breast cancer cases and is hallmarked at the morphological level by tumor cells growing in single "indian files" within a dense fibrous stroma. This phenotype can be explained at the molecular level by loss of integrity of cell adherens junctions due to mutations or methylation of the *CDH1* gene, which encodes the transmembrane protein E-cadherin (Martinez *et al.,* 1979; Borst *et al.,* 1993; Moll *et al.*, 1993; Vos *et al.,* 1997; Droufakou *et al.,* 2001). To our surprise, we found that mammary gland-specific Cre-mediated inactivation of *Cdh1* alleles in mice was insufficient to induce mammary tumors, probably because normal cells undergo apoptosis and are counter-selected when E-cadherin is lost (Boussadia *et al.*, 2002; Derksen *et al.*, 2006; Derksen *et al.*, 2011). This prompted us to investigate which co-operating oncogenic events are required for malignant transformation of E-cadherin deficient mammary epithelial cells. We have found that multifocal ILC formation is promoted by dual mammary-specific loss of E-cadherin and p53 (Derksen *et al.*, 2006; Derksen *et al.*, 2011) or E-cadherin and PTEN (Boelens *et al.*, 2016), with tumor architecture and molecular profiles closely resembling their human ILC counterparts (Table 1). However, it remains elusive which biological processes are rescued by co-depletion of E-cadherin with one of these factors. In order to identify novel candidate cancer genes and networks that collaborate with E-cadherin loss in mammary tumorigenesis, we used the *Sleeping Beauty* (SB) transposon system (Collier *et al.*, 2005; Dupuy *et al.*, 2005) to perform an insertional mutagenesis screen in *WAPcre;Cdh1*$^{F/F}$ mice (Kas *et al*., 2017). Retrieval of recurrent integrations in SB-induced *WAPcre;Cdh1*$^{F/F}$ mammary tumors identified common insertion sites in several genes, some known to be mutated in human ILC, suggesting that mutagenesis of these genes

Table 1. Characteristics of human ILC and BRCA1-associated breast cancer and the corre ponding GEMMs developed in our laboratory

In vasive lobular carcinoma	**Human**	***WAPcre;Cdh1$^{F/F}$;Pten$^{F/F}$*** **mouse model**
Morphology	Lobular	Lobular
Invasive	Yes	Yes
Grade	Low	Low
Mitotic index	Low	Low
ER expression	Yes	Yes
Molecular subtype	Luminal	Luminal
Collagen deposition	Yes	Yes
Stroma-rich	Yes	Yes
BRCA I-associated breast cancer	**Human**	***K14cre;Brca1$^{F/F}$;p53$^{F/F}$*** **mouse model**
Morphology	Ductal	Ductal
lnvasive	Yes	Yes
Grade	High	High
Mitotic index	High	High
ER/ PR expression	No	No
Molecular subtype	Basal-like	Basal-like
Genomically instable	Yes	Yes
HR-deficient	Yes	Yes

ILC, invasive lobular carcinoma; BRCA1 , breast cancer gene 1; GEMMs, genetically engineered mouse models; ER, estrogen receptor; PR, progesterone receptor; HR, homologous recombination.

leads to malignant transformation. Moreover, analysis of enriched targeted pathways and mutually exclusive insertions revealed regulation of the actin cytoskeleton as a completely novel oncogenic pathway in both mouse and human ILC.

Furthermore, recent genomic studies on collections of human ILCs have unveiled that, in addition to somatic inactivation of E-cadherin, activation of PI3K/AKT signaling appears to be a common event in this breast cancer subtype (Ciriello *et al.,* 2015; Desmedt *et al.,* 2016; Michaut *et al.*, 2016). To validate these findings, we developed GEMMs of ILC that combine mammary gland-specific ablation of E-cadherin and activation of different oncogenic *Pik3ca* or *Akt* mutants (van Miltenburg *et al*., *in prep.*). To rapidly generate breast cancer models carrying these allelic variants, we used a novel strategy for fast-track production of GEMMs, called GEMM-ESC, which is based on Flp-recombinase-mediated introduction of additional mutant alleles into the *Col1a1* locus of embryonic stem cells (ESC) derived from existing GEMMs (Huijbers *et al.,* 2014). Interestingly, the resulting mice showed rapid development of tumors with strong resemblance to human ILC in terms of morphology, gene expression and invasiveness, on which we are now

testing a panel of anticancer therapeutics to identify promising genotype-specific drug sensitivities.

Basal-like breast cancer models

Basal-like breast cancers represent a heterogeneous class of malignancies with poor clinical outcome that accounts in total for 10-15% of all breast cancer cases (Perou *et al.,* 2000; Badve *et al.,* 2011). The majority of basal-like tumors lack expression of estrogen receptor (ER), progesterone receptor (PR) and human epidermal growth factor receptor 2 (HER2), and are therefore referred to as triple-negative breast cancers (TNBC). These tumors are not targetable with hormonal therapy or HER2-inhibitors, which leaves clinicians with only few effective options for therapeutic intervention.

Approximately 50% of basal-like breast cancers display a dysfunctional BRCA pathway due to germline or somatic mutations in *BRCA1/2* or *BRCA1* promoter hypermethylation (Cancer Genome Atlas Network, 2012; Nik-Zainal *et al.,* 2016). Also a fraction of non-basal-like tumors are BRCA-deficient, mostly due to germline mutations in *BRCA2*. As these genes are crucial in the error-free repair of DNA double-strand breaks (DSBs) by homologous recombination (HR), BRCA defects are associated with chromosomal instability and hypersensitivity to DNA DSB-inducing drugs such as alkylating agents, PARP inhibitors (PARPi) and radiotherapy (Jaspers *et al.,* 2009; Bouwman and Jonkers, 2012; Barazas *et al*., *in prep*.). However, drug resistance mechanisms have been described in both clinical and preclinical studies of BRCA associated tumors, posing serious concerns as no other therapies are currently available for relapsing patients.

In order to study tumorigenesis and drug resistance mechanisms, we developed several conditional mouse models for BRCA1- and BRCA2-associated breast cancer (Evers and Jonkers, 2006; Bouwman and Jonkers, 2008). In our *K14cre;Brca1*$^{F/F}$*;p53*$^{F/F}$ (KB1P) and *K14cre;Brca2*$^{F/F}$*;p53*$^{F/F}$ (KB2P) models, mammary inactivation of *Brca1/2* is accompanied by loss of p53, as mutations in this tumor-suppressor frequently co-occur with *BRCA1/2* mutations in breast cancer (Jonkers *et al.,* 2001; Liu *et al.,* 2007). These mice develop mammary tumors after a latency period of 6-8 months, suggesting that additional mutations are required for tumorigenesis (Table 1). However, in contrast to ILC where point mutations are the most common somatic alterations, *BRCA*-mutated breast cancers are characterized by complex patterns of DNA copy number aberrations (CNAs), including translocations and gains/losses of entire chromosome arms (Vollebergh *et al.,* 2012). Using cross-species oncogenomics, we identified *MYC* amplification and *RB1* loss as recurrent CNAs in both mouse and human *BRCA1/2*-mutated breast cancers (Holstege *et al*., 2010). Exploiting the GEMM-ESC strategy, we could model conditional overexpression of MYC in our *WAPcre;Brca1*$^{F/F}$*;p53*$^{F/F}$ (WB1P) mouse model, and found

that mammary tumor development was indeed strongly accelerated compared to the original line (Annunziato *et al.*, *in prep.*). Moreover, we observed that the number of CNAs in WB1P-MYC tumors was markedly reduced compared to WB1P tumors, showing only few recurrent CNAs that most likely harbor additional cancer drivers that collaborate with MYC overexpression and loss of BRCA1/p53 in breast tumorigenesis. We are currently performing cross-species comparisons of the recurrent CNAs in WB1P-MYC tumors with CNA profiles from human breast cancers to identify candidate cancer genes, which will be validated in the WB1P-MYC model. We believe this iterative CNA profiling approach in progressively complex GEMMs will be instrumental for deciphering the key driver events in BRCA1-associated breast cancer and for uncovering novel therapeutic vulnerabilities.

Preclinical trials in BRCA-associated breast cancer models

While phase-I and-II clinical trials are mostly carried out in heavily pretreated volunteer patients who suffer from end-stage metastatic cancer, mouse models provide the opportunity to initiate treatment on naïve tumors in a clinically relevant *in vivo* setting. Treatment of mammary tumor-bearing KB1P mice with a panel of DSB-inducing agents showed heterogeneous responses between individual tumors, but also marked

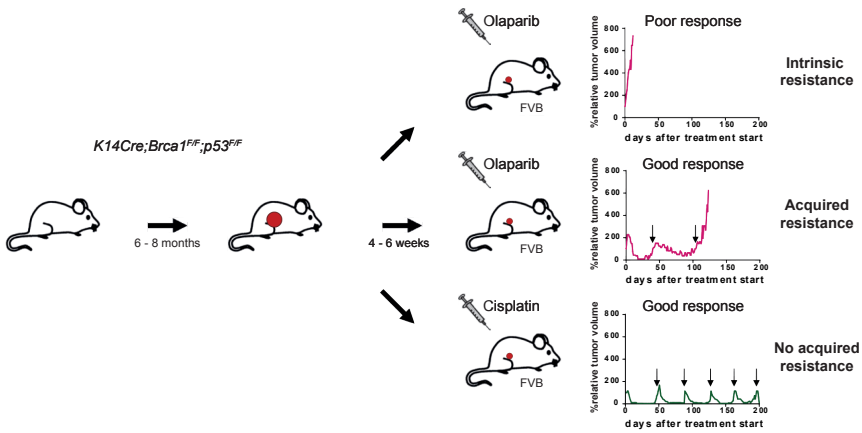

Figure 1 Large-scale intervention studies using breast cancer gene (BRCA)-deficient orthotopic allografts. Spontaneous tumors develop with a latency of 6–8 mo in *K14cre;Brca1$^{F/F}$;p53$^{F/F}$* (KB1P) or *K14cre;Brca2$^{F/F}$;p53$^{F/F}$* (KB2P) genetically engineered mouse models (GEMMs). Retransplantation of spontaneous tumors in syngeneic FVB wild-type mice highlighted intertumor heterogeneity in treatment response. In the case of olaparib, some tumors displayed intrinsic resistance, whereas others displayed initial good response followed by the emergence of acquired resistance. In contrast, resistance never developed in cisplatin-treated animals, despite multiple consecutive rounds of treatment (indicated by black arrows).

differences in tumors treated with doxorubicin or docetaxel and those treated with cisplatin (Rottenberg *et al.*, 2007). While KB1P tumors eventually developed resistance to doxorubicin and docetaxel, no acquired resistance was observed for cisplatin. Even though these tumors could never be completely eradicated by maximum tolerated dose (MTD) concentrations of cisplatin, the relapsing tumors remained responsive to subsequent treatments, resulting in a typical saw-tooth tumor response. A major breakthrough came when it was found that spontaneous KB1P and KB2P tumors could be orthotopically allografted in syngeneic mice whilst maintaining their genetic characteristics and drug sensitivity profile. This approach reduced the time to produce cohorts of tumor-bearing mice from 7-9 months to 4-6 weeks, and enabled large-scale intervention studies in which the response of a single donor to different chemotherapeutic strategies could be compared, ruling out any inter-tumor heterogeneity (Figure 1). Intervention studies with the PARP inhibitor olaparib in KB1P tumor allografts led to the development of carboplatin and olaparib switch-maintenance therapy for *BRCA1*-mutated breast cancer (Rottenberg *et al.*, 2008). This preclinical concept was confirmed in a clinical trial with olaparib maintenance therapy in *BRCA*-mutation carriers with platinum-sensitive ovarian cancer (Ledermann *et al.,* 2012; Ledermann *et al.*, 2014), and eventually led to clinical approval of olaparib (Deeks *et al.*, 2015). Similarly, intervention studies in KB2P tumor allografts showed that alkylators such as nimustine could induce complete tumor eradication (Evers *et al.*, 2010). Eradication of *BRCA*-mutated and BRCA-like cancer by high-dose alkylating chemotherapy was subsequently confirmed by retrospective analysis of data from clinical trials (Vollebergh *et al*., 2011; Vollebergh *et al*., 2014; Schouten *et al*., 2015). These and other studies illustrate the utility of GEMMs of human cancer in translational cancer medicine.

PARPi resistance mechanisms in BRCA-associated breast cancer models

In addition to accelerating preclinical trials, the KB1P and KB2P allograft platforms also enabled large-scale induction of acquired resistance to a drug of choice and subsequent identification of the underlying resistance mechanisms. The power of this approach was demonstrated with the PARP inhibitor olaparib, which was described to display selective toxicity against BRCA1/2-deficient cells (Bryant *et al*., 2005; Farmer *et al*., 2005). Indeed, KB1P tumor allografts initially responded well to treatment, but eventually relapsed and developed stable resistance (Rottenberg *et al*., 2008). This has provided a valuable collection of matched treatment-naïve and treatment-resistant tumors, which could be analyzed using next-generation sequencing or (phospho)-proteomics, thereby taking advantage of the clean genetic background of inbred mice and the known genetic profile of treatment-naïve tumors. We found that *Abcb1a* and *Abcb1b*, encoding P-glycoprotein (P-gp) efflux pumps, were up-regulated in resistant tumors and we confirmed that P-gp played an important role in mediating export of olaparib from

tumor cells (Figure 2A; Rottenberg *et al*., 2008). Resistance could be reversed when P-gp mediated drug efflux was inhibited by co-administration of tariquidar. While the clinical relevance of P-gp upregulation as cause of drug resistance remains controversial (Amiri-Kordestani *et al.*, 2012), expression of *MDR1*, the human counterpart of *Abcb1*, was recently found to be inversely correlated to olaparib response in human ovarian cancer cells (Vaidyanathan *et al.,* 2016). Such increased expression may result from complex genomic rearrangements that fuse a distant promoter to the *MDR1* gene and thereby bypass the *MDR1* promoter methylation (Patch *et al.*, 2015). The case of P-gp shows that a thorough mechanistic understanding is instrumental to combat resistant tumors, for example by co-administration of tariquidar or by switching treatment to chemotherapeutics that are poor substrates for P-gp (Jaspers *et al.*, 2013).

To dissect P-gp independent mechanisms of PARPi resistance, the KB1P mouse model was refined through germline genetic deletion of *Mdr1* resulting in the *K14cre;Brca1*$^{F/F}$*;p53*$^{F/F}$*;Mdr1a/b*$^{-/-}$ (KB1PM) model (Jaspers *et al.*, 2013). Alternatively, KB1P tumors were treated with the PARP-inhibitor AZD2461, which is a poor substrate for P-gp (Oplustil O'Connor *et al.*, 2016). PARPi resistance developed in these models despite the exclusion of P-gp related mechanisms. To identify the underlying resistance mechanisms, next-generation sequencing data from treatment-naïve and PARPi-resistant tumors were combined with data from unbiased functional genetic screens *in vitro*. Through an insertional mutagenesis screen in conditional BRCA1-knockout mouse ESCs, we found that loss of 53BP1 rescues the proliferation defect, HR deficiency and PARPi hypersensitivity of BRCA1-deficient cells by enhancing DSB end-resection (Figure 2B; Bouwman *et al*., 2010). This work from our lab and similar studies from the Nussenzweig lab (Bunting *et al.*, 2010) have led to novel mechanistic insights in DSB repair and to date several downstream effector proteins of 53BP1 have been shown to suppress HR in BRCA1-deficient cells, including RIF1 (Chapman *et al.*, 2013; Di Virgilio *et al.*, 2013; Escribano-Diaz *et al.*, 2013; Zimmermann *et al.*, 2013), PTIP (Callen *et al.*, 2013), Artemis (Wang *et al.*, 2014) and REV7/MAD2L2 (Boersma *et al*., 2015; Xu *et al*., 2015). Thorough analysis of mutational status and expression levels of 53BP1 and REV7 in PARPi-resistant KB1P(M) tumors confirmed that loss of 53BP1 or REV7 causes *in vivo* resistance to PARPi (Jaspers *et al*., 2013; Xu *et al*., 2015). Interestingly, although KB1P(M) tumors with 53BP1 loss are cross-resistant to topotecan and doxorubicin, they are still responsive to cisplatin, suggesting that platinum drugs may be a useful salvage therapy for this class of PARPi-resistant tumors (Jaspers *et al.*, 2013).

Although the majority of KB1P(M) tumors acquired PARPi resistance through restoration of HR, a substantial fraction of PARPi-resistant tumors remained defective in the formation of ionizing radiation-induced nuclear RAD51 foci (RAD51-IRIFs), which are a hallmark of HR. Moreover, when we analyzed the BRCA2-deficient KB2P tumors with

acquired PARPi resistance, none of these showed restoration of HR as measured by RAD51-IRIF assays (Gogola *et al.*, *in prep.*). This suggests the existence of alternative resistance mechanisms. It was recently shown that chemoresistance in BRCA2-deficient cells might be mediated through protection of replication forks (RF), for instance by depletion of PTIP (Chaudhuri *et al.,* 2016). It will be important to investigate if RF protection is a common feature of PARPi-resistant KB1P(M) and KB2P tumors.

In patients, mutated BRCA1 or BRCA2 proteins are often still expressed in tumors. Therefore, the large intragenic *Brca1/2* deletions present in KB1P(M) and KB2P tumors – although instrumental in genetic studies – might not fully recapitulate the biology of BRCA-associated tumors in mutation carriers. To this end, we generated several mouse models mimicking pathogenic *BRCA1* variants that are often encountered in the clinic (Drost *et al.*, 2011; Drost *et al.*, 2016). These models provided evidence that the type and location of the *BRCA1* mutation can have significant implications for the response of

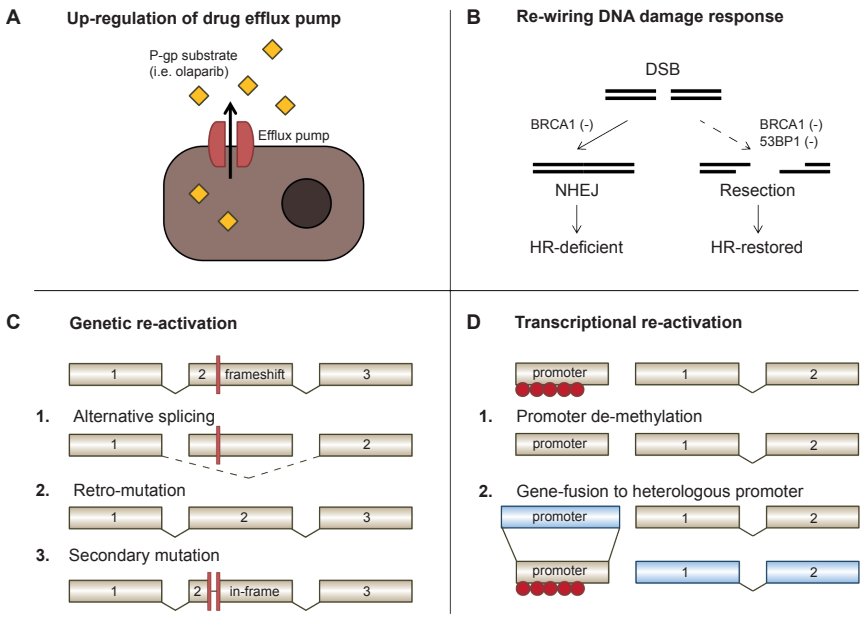

Figure 2 Overview of resistance mechanisms identified using mouse models of breast cancer gene (BRCA)-deficient breast cancer. (A) Up-regulation of drug efflux pumps (i.e., MDR1) reduces intracellular drug concentration. (B) The DNA damage response pathway can be rewired to restore homologous recombination (HR), in this case by loss of 53BP1. (C) Genetic reactivation of *BRCA1*-mutated alleles can occur because of alternative splicing, retromutations, or secondary mutations restoring the *BRCA1* reading frame. (D) Transcription of silenced *BRCA1* alleles can be restored upon promoter demethylation or gene fusions to distant promoters. P-gp, P-glycoprotein; DSB, double-strand break; NHEJ, nonhomologous end joining; HR, homologous recombination.

these tumors to DSB-inducing agents and PARPi. It was found that tumor cells harboring the *BRCA1*^{185delAG} allelic variant, which was modeled in mice by a *Brca1*^{185stop} allele, can use a downstream alternative start site leading to the expression of a RING-less BRCA1 protein (Drost *et al*., 2016). This RING-less BRCA1 protein maintains hypomorphic HR activity, which is sufficient to induce a poor response to platinum drugs or olaparib. These results illustrate the importance of testing *BRCA1* allelic variants not only for genetic counseling, but also for providing adequate treatment.

PDX models provide a solution to narrow the gap between mouse and human cancer biology and as such represent a novel *in vivo* platform for studying therapy response and resistance. Although PDX models have been relatively difficult to generate in the past, recent advances have made it possible to generate PDX biobanks covering a heterogeneous population of tumors (Hidalgo *et al*., 2014). Once the (epi)genetic landscape of a PDX model is characterized, this provides an effective tool to study the drug response of a specific tumor and to predict which resistance mechanisms might evolve during treatment. We recently demonstrated the feasibility of such an approach by treatment of PDX models of BRCA1-deficient breast cancer with alkylating agents or olaparib (Ter Brugge *et al.*, 2016). Similar to the GEMM tumors, these PDX tumors generally responded well to treatment, but eventually developed resistance. The underlying mechanism was dependent on the type of *BRCA1* inactivation: whereas therapy-resistant *BRCA1*-methylated PDX tumors frequently showed *BRCA1* promoter de-methylation, *BRCA1*-mutated tumors acquired resistance via genetic reversion through secondary mutations that restored the *BRCA1* reading frame (Figure 2C-D). These events have also been known to mediate resistance in ovarian cancer patients (Swisher *et al.*, 2008; Patch *et al.*, 2015), showing the predictive potential of PDX models. The PDX models also revealed a novel resistance mechanism involving gene-fusions that placed *BRCA1* under transcriptional control of a heterologous promoter. It is intriguing that resistance mechanisms in PDX tumors are mainly centered on re-expression of functional BRCA1 protein rather than inactivation of 53BP1 or related factors, highlighting the strong selective pressure on complete restoration of BRCA1 function when BRCA1-deficient tumor cells are exposed to DSB-inducing therapy. However, a fraction of tumors acquired resistance in the absence of BRCA1 re-expression, showing that alternative resistance mechanisms also occur in PDX models.

Taken together, these studies illustrate the power of mouse models in unraveling resistance mechanisms prior to their emergence in patients. It will be important to investigate until which extent these play a role in the clinic. This is not trivial, as they likely occur in a limited group of *BRCA*-patients and thus require careful patient selection. It is noteworthy that resistance caused by mutations in additional DNA repair genes such as 53BP1 or REV7 might expose new treatment vulnerabilities e.g. sensitivity to combined PARP and ATM inhibition (Bunting *et al.*, 2010). It will therefore be important

to determine if and how each resistance mechanism can be exploited therapeutically. Ultimately, this may provide a framework for oncologists to combat resistance in the clinic.

Non-germline GEMMs of breast cancer

Large-scale cancer genome sequencing studies and forward genetic screens have jointly boosted the discrimination between passenger and driver mutations and the identification of genetic determinants of drug sensitivity and resistance in breast cancer. The systematic translation of these long catalogues of structural aberrations into functional information requires the assessment of the pathophysiological impact of candidate gene perturbations in reliable preclinical models. This inevitably poses a practical challenge for *in vivo* validation experiments, due to the considerable costs and time requirements associated with establishing new breast cancer GEMMs. Novel technologies, especially CRISPR/Cas9-based methods, are revolutionizing the genetic engineering field by providing fast ways for precise and efficient ESC manipulation and GEMM development (Wang *et al.,* 2013). However, as sequencing expenses of human tumors keep decreasing, research will shift from testing oncogenicity of single driver alleles to investigating the impact of multiple allelic variants on tumor development and therapy response. At the same time, forward genetics strategies will evolve from genome-wide approaches based on simple gene (in)activation to more refined chemical mutagenesis and gene-based CRISPR screens capable of identifying novel hypomorphic, dominant-negative and separation-of-function mutants at the base pair level. We foresee that the number of testable hypotheses will far exceed the capacity of transgenic facilities, warranting the development of new *in vivo* platforms for systematic, multiplexed interrogation of putative cancer drivers. Ideally, such models should sort out current temporal and economical limitations of GEMM establishment, bypass extensive mouse husbandry, but also allow a high degree of manipulability and flexibility by enabling spatiotemporal control of tumor initiation and progression.

To develop such a platform for breast cancer, we explored the possibility of non-germline modeling of mammary tumors by exploiting intraductal injection in the nipple of adult female mice as a way to deliver high-titer lentiviral or adenoviral preparations to mammary epithelium and achieve somatic genome engineering. We have shown that intraductally injected lentiviruses can target tumor-initiating cells of both the basal and the luminal compartment, allowing modeling of both ILC and basal-like tumors in mice with the corresponding set of relevant predisposing alleles. For example, intraductal injection of Cre-encoding lentiviruses in *Cdh1*$^{F/F}$*;Pten*$^{F/F}$ mice induced formation of ILCs that were undistinguishable from the ILCs arising in the original *WAPcre;Cdh1*$^{F/F}$*;Pten*$^{F/F}$ model (Annunziato *et al.,* 2016). Somatic Cre delivery may more accurately recapitulate

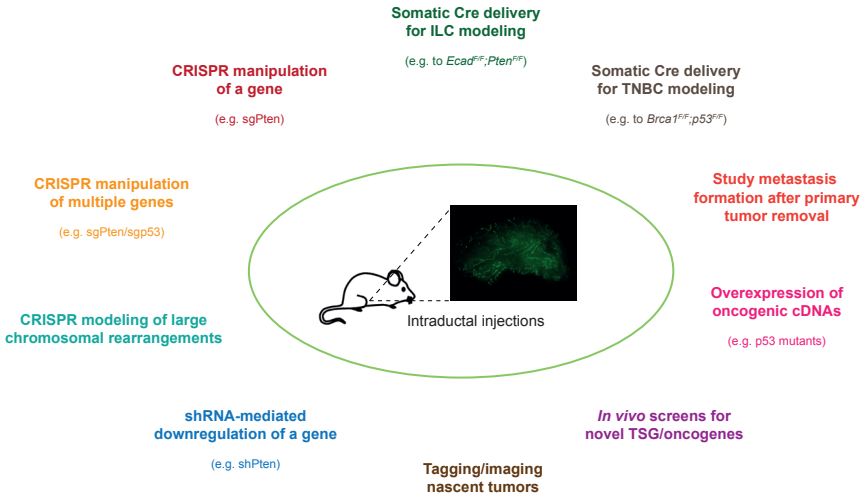

Figure 3 Multiple applications for somatic genome engineering of the mammary tissue via intraductal injection to study tumor biology. ILC, invasive lobular carcinoma; TNBC, triple-negative breast cancer; sgPten, single-guide phosphatase and tensin homolog; shPTEN, short-hairpin phosphatase and tensin homolog; TSG, tumor suppressor gene.

sporadic tumor initiation by allowing titratable and spatiotemporally controlled delivery of viruses to mammary tissue. Moreover, targeting specificity can be modulated by using viruses with cell type-specific promoters and/or post-transcriptional control elements (Tao *et al.,* 2014).

Importantly, the potential of non-germline modeling extends far beyond simple exogenous administration of Cre to established GEMMs. A diverse array of viral and non-viral constructs can be employed to achieve desired permutations of specific candidate genes even in the absence of germline conditional alleles: (a) vectors for overexpression of wild-type, truncated or mutated cDNAs; (b) vectors for shRNA-mediated downregulation or CRISPR-mediated (epi)genetic manipulation of single or multiple endogenous genes (Sander and Joung, 2016); (c) CRISPR vectors for modeling large chromosomal rearrangements (Maddalo *et al.,* 2014); (d) vectors for tagging and imaging of tumors (Figure 3). Regarding CRISPR-based *in vivo* editing approaches, we and others have shown that somatic delivery of the bacterial Cas9 protein has the considerable drawback of eliciting strong and specific immune responses in immunocompetent animals (Wang *et al.,* 2015; Annunziato *et al.,* 2016). This problem can be overcome by employing knock-in models that are tolerant to Cas9 due to constitutive or conditional expression of Cas9 or catalytically inactive dCas9-effector fusions (which allow for transcriptional silencing/activation of endogenous alleles) (Platt *et al.,* 2014; Sánchez-Rivera and Jacks, 2015). We have recently reported somatic

induction of oncogenic loss-of-function mutations in mice with mammary-specific expression of Cas9 by intraductal injection of sgRNA-encoding lentiviruses, which eventually led to ILC formation (Annunziato *et al.,* 2016).

Mammary tumor organoids

Another exciting technological breakthrough came from the possibility to derive organotypic 3D culture models of normal and malignant mammary tissue. Human and murine tumor organoid cultures retain key features of donor tumors, including cellular heterogeneity and molecular characteristics (Clevers, 2016; Fatehullah *et al.,* 2016). Compared to the laborious and time-consuming establishment of 2D cell lines, which requires adaptation to monolayer growth on plastic surfaces, tumor organoid cultures are much easier to derive, can be expanded indefinitely *ex vivo*, and upon xenografting/allografting undergo polyclonal expansion and efficiently produce tumors that preserve the cellular heterogeneity and drug response profiles of the original tumors (Duarte *et al.,* 2017). For example, we found the differential olaparib sensitivity of isogenic treatment-naïve and PARPi-resistant KB1P mammary tumors to be stable upon organoid derivation and subsequent re-transplantation. Using CRISPR/Cas9 technology, we were able to introduce *Trp53bp1* frameshift mutations in the treatment-naïve KB1P organoid line and demonstrate that this permutation rendered the organoid-derived tumors refractory to olaparib. We are exploiting the KB1P tumor organoid platform to test additional candidate drug resistance genes for their *in vivo* relevance, including candidates retrieved from forward genetic screens and from sequencing of drug-resistant tumors (Figure 4). Moreover, given the short latency period and polyclonal tumor outgrowth, GEMM and PDX tumor organoids are particularly amenable for *in vivo* genetic screens using shRNA, CRISPR, CRISPRi and CRISPRa libraries.

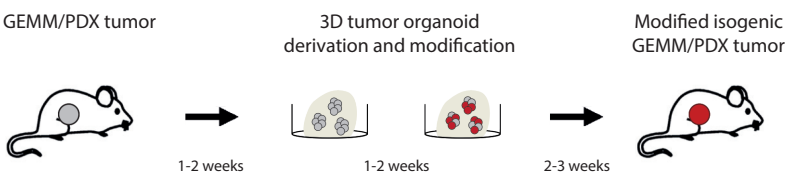

Figure 4 3D tumor organoid cultures can be rapidly derived from established genetically engineered mouse model (GEMM)/patientderived xenograft (PDX) tumors, modified ex vivo with desired genetic permutations and retransplanted in mice to evaluate *in vivo* treatment responses.

Conclusions

A number of known and unknown biological discrepancies inevitably exist between mouse models and humans. Moreover, refinements in mouse modeling should be compliant with practical and ethical issues associated with model establishment. Nevertheless, the systematic and synergistic deployment of complementary *in vitro* and *in vivo* platforms (GEMMs, PDX models, organoids, non-germline models) is envisioned to provide a quantum leap in the oncology arena and in breast cancer research in particular. Cutting-edge mouse cancer clinics will enable so-called co-clinical trials, in which clinical studies will be paralleled by preclinical intervention studies in mouse avatars. This will allow clinicians to infer in real-time genotype-specific drug response profiles from mouse models and design more effective and long-lasting patient-tailored treatment schemes. The emergence of drug resistance is an invariable and intrinsic consequence of Darwinian tumor growth dynamics, but instead of "whack-a-mole" treatment schedules, co-clinical trials could assist in the design of more sophisticated and personalized regimens in which tumors are forced through evolutionary bottlenecks that render them exquisitely sensitive to secondary therapies. Re-iteration of this adaptive process is possible only by the use of ever-smarter mouse models, which will ultimately lead to improved long-term management of this devastating disease.

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