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REVIEW

The next generation personalized models to screen hidden layers of breast cancer tumorigenicity

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Abstract

Background Breast cancer (BC) is a challenging disease and major cause of death amongst women worldwide who die due to tumor relapse or sidelong diseases. BC main complexity comes from the heterogeneous nature of breast tumors that demands customized treatments in the form of personalized medicine.

Review of the literature and discussion Spatiotemporally dynamic and heterogeneous nature of BC tumors is shaped by their clonal evolution and sub-clonal selections and shapes resistance to collective or group therapies that drives cancer recurrence and tumor metastasis. Personalized intervention promises to administer medications that selectively target each individual patient tumor and even further each colonized secondary tumor. Such personalized regimens will require creation of in vitro and in vivo models genuinely recapitulating characteristics of each tumor type as initiating platforms for two main purposes: to closely monitor the tumorigenic processes that shape tumor heterogeneity and evolution as the main driving forces behind tumor chemo-resistance and relapse, and subsequently to establish patient-specific preventive and therapeutic measures. While application of tumor modeling for personalized drug screening and design requires a separate review, here we discuss the personalized utilities of xenograft modeling in investigating BC tumor formation and progression toward metastasis. We will further elaborate on the impact of innovative technologies on personalized modeling of BC tumorigenicity at improved resolution.

Conclusion Heterogeneous nature of each BC tumor requires personalized intervention implying that modeling breast tumors is inevitable for better disease understanding, detection and cure. Patient-derived xenografts are just the initiating piece of the puzzle for ideal management of breast cancer. Emerging technologies promise to model BC more personalized than before.

Keywords Breast cancer · Personalized medicine · Xenograft · Modeling · Tumorigenesis · Metastasis

Abbreviations

AI	Artificial intelligence
BC	Breast cancer
BC-CSCs	Breast cancer–cancer stem cells
CSCs	Cancer stem cells
EMT	Epithelial-to-mesenchymal transition
GEMMs	Genetically engineered mouse models
iPS	Induced pluripotent stem
nGEMM	Non-germ line GEMM
NGS	Next generation sequencing
PDX	Patient-derived xenograft
PM	Personalized medicine
ts	Tumor suppressor

Introduction: molecular insight to breast cancer tumor evolution

Two mutually inter-convertible models have been offered to explain tumorigenesis and tumor progression to heterogeneity: the clonal evolution model and cancer stem cell (CSC) model (Fig. 1a). Genetic inhomogeneity of breast cancer (BC) tumors among others [1, 2] has been fully appreciated with the advent of next generation sequencing (NGS) that, for the first time, allowed tumor genome to be spatiotemporally mapped [2]. The level of heterogeneity varies

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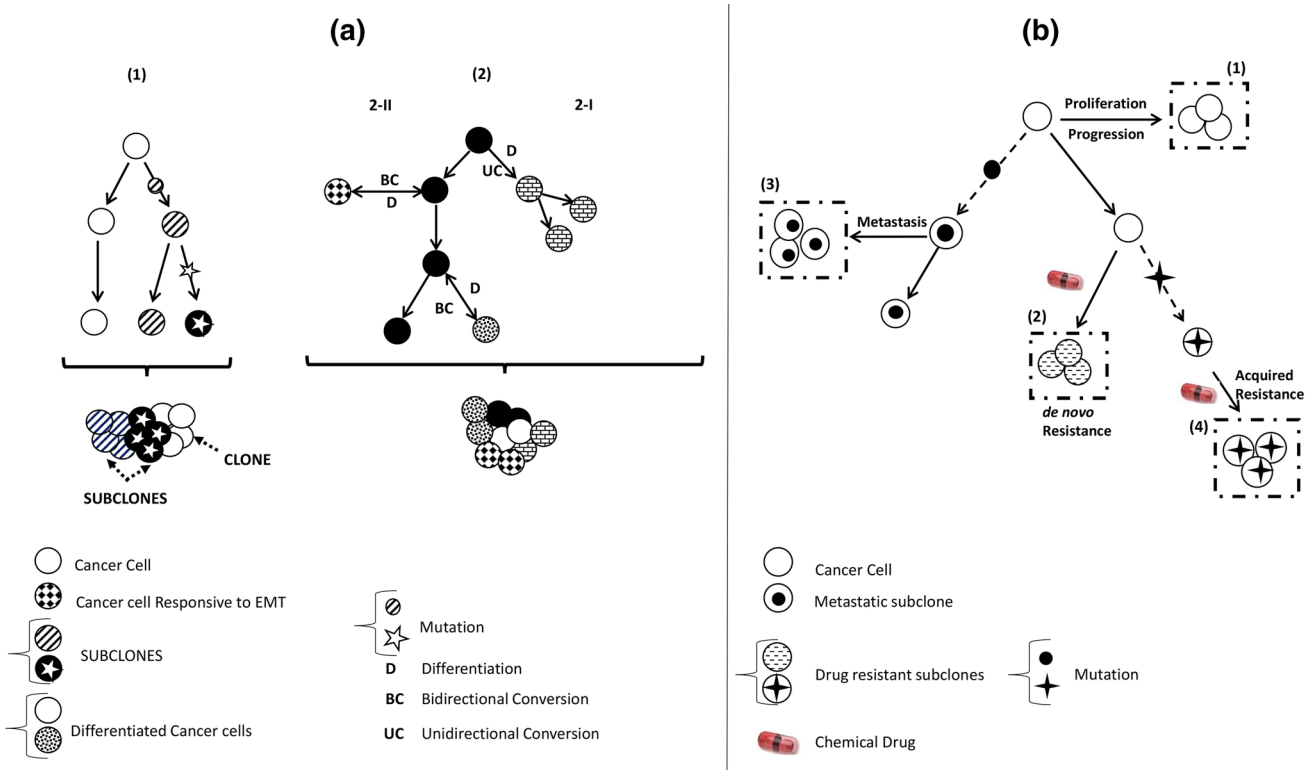

Fig. 1 Cancer heterogeneity and evolution. **a** Hypothesized models behind cancer heterogeneity. (*1*) Clonal evolution model states that a cancer cell reproduces to identical daughter cells and further undergoes mutations to produce cells with altered phenotype. Further mutations of these initial clones produce new subclones each with its distinct phenotype and characteristics for growth, drug response, EMT potential and metastatic power. (*2*) Cancer stem cell (CSC) model indicates that cells with stem cell characteristics from the epithelium begin dividing to produce different phenotypes (clones and subclones) to generate a heterogeneous cancer tumor. In *Classical Model* (*2-I*), a CSC undergoes unidirectional conversion to produce a differentiated cancer cell with no stem cell abilities, but in *Plastic Model* (*2-II*), CSCs undergo bidirectional conversion to generate various clones/subclones with distinct properties and potentials such as their responsiveness to EMT. **b** Different fates of clones and subclones. A cancer cell proliferates to produce a bulk of identical cells and also subclonal cells that gain potential to overgrow (*1*), resist therapy (*2*), metastasize (*3*), etc. Some clones made from the initial cancer cell show inherent resistance to chemotherapy (de novo resistant), (*2*) while according to NGS findings, a rare subclone emerges at early steps of tumorigenesis (upon genomic instability of initial cancer cells), proliferates and becomes more genetically distinct and ultimately escapes chemotherapy (acquired resistant) to drive relapse and metastasis (*4*). Photoshop CC 2017 and Microsoft Office 2013 PowerPoint programs were used to create this and subsequent figures

among tumor types, and from initial steps of tumor formation to drug resistance, recurrence and metastasis. The main sources of intra-tumoral heterogeneity include sub-clonal evolution driven by mutations at different time points during tumor evolution [3], epigenetic mechanisms such as DNA methylation, chromatin remodeling and post-translational modification of histones [4], genomic changes including copy-number variation as well as chromosomal instability and alterations [5].

A BC tumor originates from a single normal epithelial cell and ends up with a clinically aggressive metastatic cancer. This reflects succession of several steps accompanied by many genetic-epigenetic events in parallel that drive multiple cellular populations with distinct or changing phenotypes [6]. During this whole process, many cellular sub-clones follow some previous populations with some persisting, some declining and some new ones emerging. It is the clonal outgrowth of some subclones driven by either an activated growth pathway, inactivated tumor suppressor pathway, or combinations that ultimately shape the heterogeneous cellular landscape of the tumor [7]. A study by the Cancer Genome Atlas Network applied microarrays and NGS on a large cohort of BC cases and reported a great diversity exists among distinct BC molecular subtypes with both genetic lesions and epigenetic mechanisms to blame [8]. Identified molecular changes included subtype-specific and high rate mutations in *TP53* tumor suppressor gene or in *PIK3CA* gene, altered gene copy number due to amplification of *PIK3CA* gene and chromosomal deletions in *HER2, TP53* and *MAP2K4*.

Breast tumor modeling for personalized management

A comprehensive project called METABRIC investigating copy-number and expression profiles of nearly 1000 primary BC samples identified top 1000 candidate driver genes with enriched oncogenes and tumor suppressor genes among them [9]. The samples were divided into several integrative clusters in which gene amplifications and multi-driver mutations were identified [10]. The consensus was that different levels of intra-tumoral heterogeneity exist in each cluster in individual tumors and that such a diversity is a biomarker of disease specific survival.

Since genomic instability of BC cells is the suspected driving force behind tumor heterogeneity, nearly every individual BC patient must be investigated for properties of her BC-CSCs and expression alterations of her transcriptome including various noncoding RNAs. The findings will then have to be applied for tumor characterization, subtype specification and eventually patient stratification, pharmacogenomic studies and drug combination efficacy testing [11].

A recent wave of attempts to apply innovative techniques and technologies promises to give rise to valuable successes in resolving tumor heterogeneity puzzles. These include and will be discussed in this review, multi-region sampling and sequencing, analysis of body fluid tumor DNA, precision detection mediated by artificial intelligence, tumor genome editing and more. But as it will be shown, for the implementation of the full potential of such innovations, we still require a duplicate model of each given tumor as solid platforms for translational studies and personalized decision for cure.

Cell lines are applied as initial tools for cancer research; however, they cannot form personalized models as they fail to imitate critical features of a tumor and its microenvironment. Computer-assisted 3D simulation of tumors and 3D organoids are steps ahead in addressing the phenotypic limitations imposed by 2D nature of cell lines. Induced pluripotency or genome editing tool boxes are developed to dissect the tumorigenic process each tumor may have experienced for its development (see below). However, these approaches are limited by producing or relying on partial sampling/modeling and, at the end, each one needs a more comprehensive animal model of tumors to produce a full picture of tumor development in basic studies and then for co-clinical personalized trials.

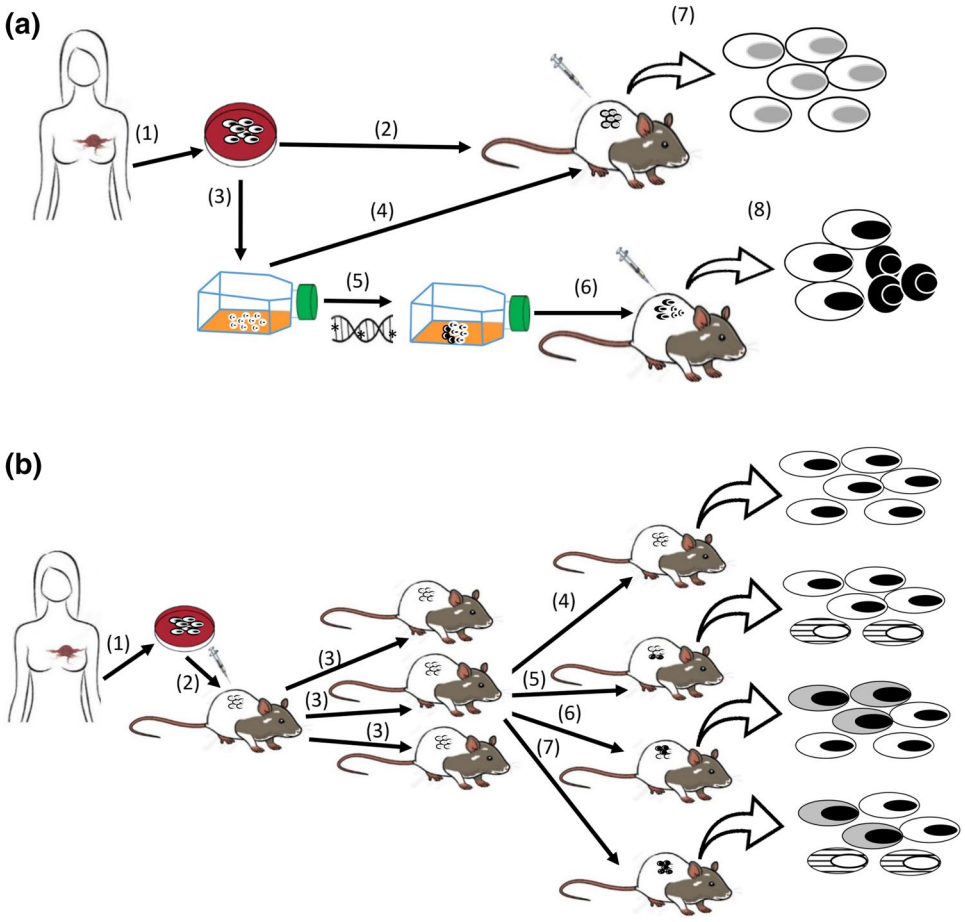

Fig. 2 Xenograft production and xenograft clonal evolution. **a** Creation of PDX. Tumor sample is isolated from patient candidate (*1*) and used to both inject into the immune-suppressed mouse (*2*) and isolate primary tumor cells (*3*). These cells can be directly injected (*4*) or subjected to DNA modification (**5**) before injection (*6*). As a result of injections, xenograft tumors are developed that contain plain (*7*) or genetically modified tumor cells (*8*). **b** Clonal evolution of xenografts. Tumor cells are isolated (*1*) and injected to generate G0 xenograft (*2*) and propagate to G1 (*3*). Subsequent propagation of G1 mice can generate G2 xenografts that could be G1 duplicates (*4*), new sub-clones (*5*), mouse-specific clones (*5*) or carry mixed multi-clones (*6*)

Mouse models carry advantages of easy handling, short gestation period and low maintenance cost (Fig. 2) [12, 13] and are therefore the most widely applied in vivo systems to study cancer [14]. Besides, genetic manipulation of mice that allows expression induction, recognition targeting and spatiotemporal gene regulation makes it possible to recapitulate human diseases [15]. Early passage patient-derived xenograft (PDX) models ensure retaining genomic and gene expression patterns of the parental tumor for a few passages and so are preferred ones for molecular investigations. Besides, more aggressive tumor types with a higher proliferative index emerge by passaging xenografts [16]. As for BC, the PDX models of all subtypes have been generated in mice. As part of global efforts on modeling BC drug resistance, our group generated PDX models specifically for anti-HER2 trastuzumab resistance and subjected them to molecular analyses [17].

Application of PDX models for personalized breast cancer management

Preservation of human breast cancer tumors by PDX

An ideal model for personalized medicine applications has to reflect and retain the original tumors' biological integrity, heterogenic diversity, and genetic and genomic alterations. Molecular genetic and metabolic preservation have been established to high extent from the original tumor subtypes to their PDX models [18, 19]. In fact, several microscopic, cellular and molecular layers of PDX models outlined below have shown to be indistinguishable duplicates of those in the parental tumor:

A. Cellular preservations that include the fine tissue structure and microscopic details such as gland architecture, mucin production or cyst development.
B. Molecular recapitulations that include staining various markers such as estrogen receptor (ER) and HER2 expression of the source tissue, genomic rearrangements, copy number alterations, mutation profiles and variant allele frequencies [20].

High-level fidelity and stability have been also reported at both transcriptomic and proteomic levels across transplantation generations [21]. Indeed, such recapitulation of original tumor features at all the levels mentioned above has been confirmed by the comprehensive modeling project METABRIC that created and examined a biobank collection of PDX [19]. Since PDX preserve variant allele frequencies, their passage can be used to transplant clonal representation. Furthermore, intrinsic BC phenotypes are preserved by PDX tumors that include single mutations as well as mutation enrichment patterns observed in the metastatic tumors [22]. Differential analyses of mutation frequencies and structural variation patterns from patient tumor metastasis to xenograft modeling can identify the minority population within the original tumor that makes it metastatic [22].

In addition to ordinary PDXs, two initiatives have been lately launched to address intra-tumoral heterogeneity and inter-tumoral variability: "Co-clinical Trials" and the use of "Mouse Avatars". The first initiative utilizes genetically engineered mouse models (GEMMs) to assist patient therapy in ongoing clinical trials [23], but the second relies on PDX modeling in screening appropriate chemotherapeutic agents [24].

Clonal stability and sub-clonal selections: studies on PDX

Tumor clones emerge as tumors evolve parallel with successive genetic/epigenetic alterations. Several *de novo* or acquired intra-tumoral changes then occur at later stages of tumor passaging, sampling and xenografting to shape sub-clonal tumor evolution (Fig. 1b). Clonal and sub-clonal diversities are measures of tumor dynamics with major impact on tumor outcome and so are subject of intense preclinical studies in translational cancer medicine [2].

Studies based on clustering tumor cells into sub-clones each carrying a specific mutation found human-to-mouse and mouse-to-mouse clonal dynamics largely remains stable [25]. Also clonal dynamics was found similar in independently engrafted mice regardless of their prevalence in the originating human tumor sample, in line with previous observations that clones follow deterministic evolutionary trajectories [25]. Despite these and other reports indicating preservation of original molecular structure by PDXs for generations [19, 21], minor deviations, however, have been reported mainly in genes that belong to human stroma and extracellular matrix [26]. Likewise, the METABRIC project detected a model-specific drift in engrafting that was influenced by a few patient- and tumor-specific determinants and the immunosuppressed environment of tumor growth in the mouse host [19, 25].

Tumors can indeed form in a complex nonlinear branching model, as shown by studying leukemia at single cell levels [27]. Therefore, measurement of genomically defined clonal population dynamics can be highly informative for functional studies. For example, genomic analysis of a PDX model for a basal-like primary BC tumor showed the xenograft retains primary mutations but further carries *de novo* mutations emerging in metastatic lesions [22]. In this study, the PDX was indeed genetically closer to the metastatic stage of the tumor. In addition, comparison of PDX models at different time points showed that variation at single nucleotide level can occur during xenograft passaging

[28]. Subsequently, Ben-David et al. analyzed landscapes of aneuploidy and copy number alterations in multiple human cancer models and found substantial divergence from parental tumors during PDX passages, as indication of mouse-specific tumor evolution [29]. Therefore, the risk of clonal selection, loss of tumor integrity and altered human identity cast doubt on the notion that PDX models are highly stable and point to their genomic instability when compared to their parental tumor.

The question of genomic instability

Sub-clonal and mouse-specific evolution of PDX tumors demand early passages to be applied in clinical trials. In fact, tumor sampling carried out from a single patient at different time points has indicated multiple sub-clones [11] and hypermutable loci present within the tumor [30]. Therefore, to examine the initial steps of tumorigenesis, both primary and metastatic models have to be produced in pair to allow thorough investigation of all tumorigenic steps from the same patient.

It appears that both preexisting alterations, especially at the early stages of PDX derivation and propagation, and acquired changes during model tumor development contribute to the genomic instability. PDXs show strong clonal dynamics in revealing chromosomal aberrations undetectable in their original tumors [29], presumably due to distinct selection pressures that may exist between patients and animal models. These deviations from parental tumor composition can be partially addressed for clinical applications using multiple cell line models from a single primary tumor because such lines may act better than a single PDX model in reproducing more of the original genomic landscape and its heterogeneity. Such multiple modeling will likely capture not only copy number alterations but also other molecular changes that can occur throughout model propagation [29].

PDX produced from stable tumors show exceptional gene signatures related to tumor invasion and metastasis. A study on the PDX models correlated enhanced tumor cell proliferation and MYC expression with metastatic progression [31]. According to this study, tumor progression toward metastasis can be reduced upon inhibition of cyclin-dependent kinases [31].Yang and co-workers have outlined the methodology of establishing PDX models for tumor metastasis [32].

At genomic levels, metastases have been shown to evolve independently from primary tumors, presumably from common ancestral sub-clones hidden in the primary tumor but detectable at later stages. Also, distinct metastatic sites of the disease show diminished levels of heterogeneity compared to their primary tumors [33]. Therefore, distinction between the genetics of metastatic tumors from that of their primary ancestors both in humans and PDX models needs to be done carefully. As suggested by Ben-David et al., the PDX models and their matched primary ancestors may have different genetic compositions that will impact personalized management of the tumors and drug discovery using tumor models in particular the avatar mice [29].

Precision modeling of breast cancer using technological innovations

The revolutionizing potential of novel tools and technologies is changing the pace of personalized cancer research including cancer modeling. A number of these innovations in respect of their application in tumor modeling are briefly outlined below:

Sampling technologies: cancer evolution detection

New liquid sampling protocols are being developed alongside multiregion sequencing to address both spatiotemporal tumor heterogeneity and profound tumor dynamics. Such comprehensive methodologies are based on the dynamic concept of tumor evolution that requires real-time analysis of both clonal and subclonal tumor alterations.

The biological samples of a patient often contains circulating tumor DNA (ctDNA) [34] that can be relied upon for sensitive analyses because ctDNA molecules truly reflect changes specific to the tumor [35, 36] and their short half-life allows tracing changes at the level of allelic frequencies. This sensitivity of the body liquid sample allows early-stage detection of ctDNA molecules with both trunk and branch mutations [37–39] and the presence of mutant oncogenes in treated metastasis as indication of resistant subclones well ahead of monitoring relapse by imaging [40, 41]. Such approaches have detected multiple *KRAS* and *NRAS* mutations (polyclonal drug resistance) in metastatic breast tumors [42]. Both whole genome and exome sequencing of liquid biopsies from BC and other cancers identifies markers of drug resistance and even chromosomal alterations in both number and structure [43, 44]. Changes in receptor tyrosine kinases that have pivotal role in BC tumorigenesis and drug resistance can be monitored by analyzing liquid biopsies as in the case of *HER2* amplification upon trastuzumab treatment of a patient with gastric cancer [45]. Garcia-Murillas et al. have shown in early-stage BC that mutation tracking in ctDNA increases sensitivity of predicting relapse and, by detecting genetic events of minimal residual disease, predicts metastatic relapse more accurately than sequencing of the primary cancer [46]. Relevant to tumor modeling and in particular in co-clinical trials, PDX can provide backup to liquid biopsies alongside patient samples.

Artificial intelligence: tumor evolution prediction and modeling

Artificial intelligence (AI) is an algorithm-based strategy of simulating intelligence programmed in a man-made machine for correction and rationalization of actions. deepCODE is an AI machine that has been used in xenograft models of human tumors to classify BC tumors by identifying tumor subtypes with maximum possible accuracy [47].

An AI-based method has shown its utility in analyzing multi-region sequencing data that were collected from numerous patients. The method identified previously unknown repeated evolutionary trajectories in subgroups of patients [48]. The AI technology has also been used for single mutation detection [49], tissue-specific gene expression [50] and expression of genes shared between xenografts and tumors of origin [51]. These advances in the AI applications have significant implications in investigating tumor biology and tumor modeling.

The technology alone can be applied to monitor molecular events in the course of tumor formation in PDX models and so to confirm the outcomes of human tumor-based investigations on BC tumor evolution. Such cross-comparison can reveal discrepancies of tumorigenesis between the two species. Post-transplantation changes in gene expression patterns are major sources of such molecular discrepancies. A novel computational method incorporating several machine learning algorithms, including Monte Carlo feature selection, random forest and rough set-based rule learning, was applied to 174 human tumors to examine 32 selective genes for constructing a prediction model [52]. The system detected seven interpretable interactions within the informative genes that were verifiable by previous experimental studies. Spatiotemporal precision offered by AI technologies could change the pace of tracing specific gene expression patterns by relying on generously available tissues of PDX models. Besides, AI approaches now offer precise introduction of genes into specific tissue locations and so single gene delivery (Transposase Technology, http://emag.medicalexpo.com/ai-could-revolutionize-gene-therapy/) into PDX model tissues could further expand our capabilities in investigating revolutionary patterns of spatiotemporal gene expression.

Simulation of tumorigenic pathways using *in silico* approaches [53] and AI machines such as TUGROVIS (https://hci-kdd.org/project/tumor-growth-machine-learning/) will further allow creation of more personalized models elegantly reflecting the diversity of each individual human tumor. Such models will be amenable to specific molecular manipulations by genome editing or innovative arms of optogenetics (see below) and therefore will find applications in human-mouse-matched co-clinical trials that heavily depend on mouse models.

Organoids: three-dimensional cancer modeling

Apart from mathematical or virtual simulation of tumor growth (like in the MAXIMA project, http://maxima-tuv.eu/, that models tumors to study advanced X-ray breast imaging techniques), tumor tissues can be grown in 3-dimensional modes in culture dishes. Organoids are 3D structures produced in suspension cultures which disallow cell attachment for 2D growth [54]. Organoids are suitable for engineering of tissues that have histological and functional similarity to their original in vivo counterparts and so can facilitate genuine modeling of cancer for drug screening and replacement tissue/organ generation. Organoid cultures might provide better control of cellular milieu than PDX models, better spatial organization of cell types and superior mimicry of in vivo cell behavior to 2D cultures. They might also be advantageous for screening drugs at large scale, ethical concerns, time and cost for PDX formation [54–56].

Genome editing: molecular cancer surgery

Recent RNA-based genome editing systems notably the CRISPR/Cas9 introduce double-strand breaks in specific sites of DNA [57]. Recapitulation of cancer cell events such as chromosomal translocations [58–60], examination of various aspects of tumorigenicity by introducing or reversing specific mutations and cancer cell genome labeling in combination with DNA barcoding techniques [61] are some of the recent developments in the utility of CRISPR/Cas9 editing in cancer research. Added to the list is CRISPR/Cas9-mediated simulation of progressive and stochastic changes that happen as tissue or tumor cells divide that ultimately allow inferring and mapping the lineage relationships and dynamic event histories from the colonized cell mass [62].

The versatility of CRISPR gives rise to model cell lines and PDX carrying desired mutation(s) toward global dissection of oncogenic signaling pathways and analysis of epistatic interactions. CRISPR/Cas9, combined with conditional expression systems, generates an unprecedented speed and un-paralleled spatiotemporal precision for introducing genetic changes within specific loci in cancer models specially genetically engineered mouse models (GEMMs) and non-germ line GEMMs (nGEMMs) as valuable tools in studying many stages of tumorigenesis from initiation to progression, drug resistance and metastasis [63, 64]. Such a versatile technique allows for large-scale functional studies in tumorigenesis and so bypasses many barriers and limitations carried by traditional knock-in or knock-out approaches and tedious transgenic strategies. Mice with conditional Cre–*loxP*-based alleles and reporter alleles or those carrying small deletions in their genomes have been created using the CRISPR/Cas9 system [65]. Smart CRISPR/Cas9 combinations with innovative tools or approaches have indeed

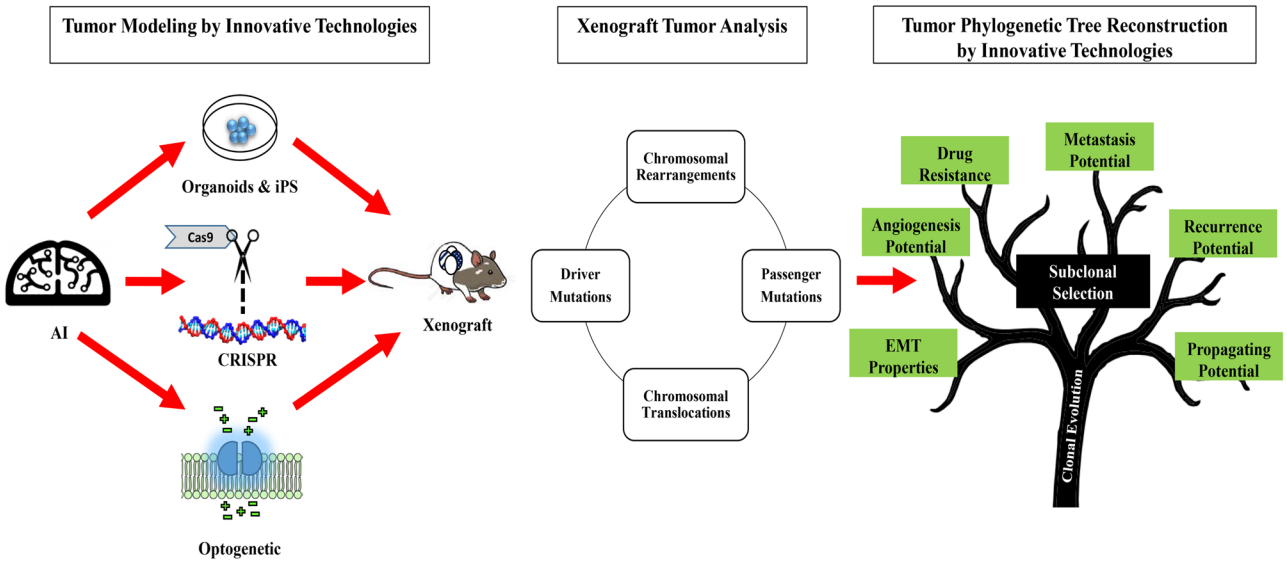

Fig. 3 Personalized Tumor Re-modeling and Management Using Innovative Technologies. This schematic figure shows that future models will be created by innovative technologies. Once created, the technologies will help to dissect the models and produce required data for construction of the personalized phylogenetic tree to be used for personalized management of the tumor. The central role of artificial intelligence in guiding many technologies to implement precision cancer medicine is emphasized

produced *ex vivo* models of human tumor malignancies by disrupting single or multiple genes [60, 66, 67] and *in vivo* models with either two *ts* genes edited inducibly using hydrodynamic transfer technique [68] or three *ts* genes using lentivirus vectors [69, 70]. Likewise, lentiviruses [71] and adenoviruses [72, 73] co-carrying CRISPR and sgRNAs for target genes generated models of oncogenic chromosomal rearrangements in vivo.

Tumor models in return provide spatiotemporal opportunities for the CRISPR/Cas9 genome editing to analyze endogenous gene expression with lineage reconstruction in the same tumor cells. Such studies on first generation PDX could create next generation models so step-wise analysis of tumor models reveals hidden molecular trajectories and allows the tumorigenic pathways to be mapped within each individual tumor eventually shedding light on the original tumor for personalized management of each patient. In addition, CRISPR/Cas9 combined with iPS will pave the ground for generating more versatile and informative models of cancer and allow the impact of gene targeting approaches on personalized tumor therapy to be thoroughly evaluated [74].

Personalized breast cancer modeling: what we have and what is on the horizon?

The first step to fully address challenges that translation of personalized cancer medicine to clinical settings faces and to stratify patients into appropriate personalized trials is to generate tumor models with improved engraftment rates at reduced timelines so to confidently correlate PDXs to their original tumors and integrate drug screening. The PDXs facilitate real-time and successive analysis of BC tumorigenesis and can be relied upon for repeated sampling and multiregion sequencing at stages not possible for patient sample collections. Early liquid, biopsy and engraftment sampling will ensure monitoring early events behind BC tumor resistance and recurrence. The phylogenetic tree of a single tumor can be drawn from multiregion sampling of the tumor to reflect its evolutionary dynamics. Clonal development constitutes the trunk, whereas subclonal genetic changes form the branches of this tree.

Cell and animal models can both be developed by such phylogenetic trees and, in return, help to elucidate unknown or hidden branches using tumor genome editing and even optogenetic toolboxes. This reciprocation is much needed for well-planned next generation models to practice individualized tumor management in the context of precision medicine.

The versatility of AI can play a central role by determining the framework for other tools and techniques (Fig. 3). Changes in tumor genome detectable using AI technology can be relied upon for predicting PDX behavior during tumorigenesis and for personalized tumor modeling. Furthermore, AI can improve speed and accuracy of genome editing in mutating cancer genes or support optogenetics in cancer reprogramming toward personalizing the models. The technologies are also applicable to identifying CSC markers in all steps of BC development at both intra-tumoral and systemic levels. Optogenetics, fed by AI-produced data, is

now applied as a robust enlightening tool for tumor studies and, together with genome-editing tools and other AI-based strategies, promises to revolutionize BC metastasis detection. The expansion of genome editing, on the other hand, will be critical for addressing mouse-specific deviations that may occur in PDX tumors of BC and for restoring tumors' originality so the models can be trustworthy for further use.

Finally, combination and customization of such advanced tools with avatar and co-clinical initiatives will be expectedly vital for clarifying molecular pathology of BC tumors, accurately predicting clinical outcome, discovering powerful biomarkers and serving patients who are unfit for clinical trials. They will also address polypharmacy (meaning concurrent use of various medications beside the anti-tumor trial) and resolve patient drop-out due to drug toxicity and therapy resistance, all measures necessary for personalized management of BC.

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Compliance with ethical standards

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

Ethical approval This article does not contain any studies with human participants or animals. The study was approved by the institutional review board for a retrospective chart review.

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