Published in final edited form as:

Pharmacol Ther. 2016 September ; 165: 79–92. doi:10.1016/j.pharmthera.2016.05.007.

Personalized *in vitro* Cancer Models to Predict Therapeutic Response: Challenges and a Framework for Improvement

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Abstract

Personalized cancer therapy focuses on characterizing the relevant phenotypes of the patient, as well as the patient's tumor, to predict the most effective cancer therapy. Historically, these methods have not proven predictive in regards to predicting therapeutic response. Emerging culture platforms are designed to better recapitulate the *in vivo* environment, thus, there is renewed interest in integrating patient samples into *in vitro* cancer models to assess therapeutic response. Successful examples of translating *in vitro* response to clinical relevance are limited due to issues with patient sample acquisition, variability and culture. We will review traditional and emerging *in vitro* models for personalized medicine, focusing on the technologies, microenvironmental components, and readouts utilized. We will then offer our perspective on how to apply a framework derived from toxicology and ecology towards designing improved personalized *in vitro* models of cancer. The framework serves as a tool for identifying optimal readouts and culture conditions, thus maximizing the information gained from each patient sample.

Keywords

Adverse outcome pathway; Chemotherapy sensitivity and resistance assays; Targeted therapy; Microenvironment; Personalized medicine

1. Introduction: Need for personalized medicine, current approaches and *in vitro* models

Classically, patients have relied on systemic treatments and invasive procedures such as chemotherapy, radiation therapy, and surgery to combat cancer. Choosing the right cancer treatment is difficult because limited tools, money and time are available to guide this

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Conflict of Interest Statement: David J. Beebe holds equity in Bellbrook Labs, LLC, Tasso, Inc., Stacks to the Future, LLC and Salus Discovery, LLC.

decision. The therapeutic index for most cancer treatments is extremely narrow, requiring a balance between drug efficacy and toxicity tolerance. In a given population, drug efficacy and tolerance can differ greatly between individuals and even across an individual tumor (Gerlinger et al., 2012; Leonard, Williams, Tulpule, Levine, & Oliveros, 2009). The mechanisms underlying cancer development and progression vary drastically from patient to patient and a cure for one patient can be ineffective or harmful to another. Researchers have shifted from trying to identify silver bullet “cures” for a given cancer to attempting to find solutions to combat patient specific cancer subtypes or in other words personalized medicine (Ginsburg & McCarthy, 2001; Veer & Bernards, 2008).

Personalized medicine encompasses both tumor and non-tumor systemic patient phenotypes that contribute to the effectiveness of a treatment/therapeutic (Bartlett et al., 2014; Chin, Andersen, & Futreal, 2011). Human populations can exhibit profound systemic differences in drug disposition, which can also contribute to therapeutic response. Identifying polymorphisms in genes that encode for drug metabolizing enzymes can help determine if a patient will experience adverse effects or even no effects in response to a drug (Johansson & Ingelman-Sundberg, 2011; Tomalik-Scharte, Lazar, Fuhr, & Kirchheiner, 2007). For instance, individuals that carry the uridine diphosphate glucuronosyltransferase (UGT) 1A1*28 gene variant produce less of the UGT1A1 enzyme and are at high risk for irinotecan associated morbidity and mortality (Bosch, Meijerman, Beijnen, & Schellens, 2006; Iyer et al., 2002).

Assessment of tumor cells themselves provide a lens into susceptibilities of a tumor to targeted therapies. One method to evaluate tumor cells is through profiling the tumor’s DNA, RNA, or protein, to identify molecular biomarkers that are predictive of patient response. A second method is to integrate tumor cells into chemosensitivity and resistance assays (CSRAs), a term used to describe an *in vitro* functional assay that measures response to a drug *ex vivo*, which will be discussed in detail later. Investigating the molecular profile of cancer cells to identify biomarkers can help predict drug resistance, and have identified gene expression profiles that correlate with cancer recurrence after specific drug treatments (Chang et al., 2003; Estes, Lovato, Khawaja, Winter, & Larson, 2007; Marchionni et al., 2007). Small molecule kinase inhibitors such as gefitinib or erlotinib are the suggested drug treatment for lung cancer patients whose tumors have mutations in epidermal growth factor receptor. However, if those patients also have mutations in KRAS they are at high risk for gefitinib and erlotinib resistance (Pao et al., 2005). Gene expression combined with protein secretion profiles of cancer cells also identify biomarkers for targeted therapies (Dias-Santagata et al., 2010; Harris et al., 2007; Sawyers, 2004). One study of patients with advanced cancer including over 15 different tumor types found that patients who underwent targeted therapy had a significant increase in overall response rate, time to treatment failure, and survival duration (Tsimberidou et al., 2012). While molecular profiling of the tumor is a powerful approach, the focus of this review is to discuss the technological advancement of CSRAs and methods to improve their clinical use.

Beyond tumor cells themselves, some cancer therapeutics do not target the tumor alone but also the associated microenvironment. In breast, colorectal and prostate carcinomas, stromal components actively participate in cancer development and are continuously modified as the

disease progresses. It has been demonstrated that tumor-associated stroma undergoes extensive changes in gene expression and that a stromal transcript signature correlates with histological tumor grade (Calon et al., 2015; Chang, et al., 2003; Tuxhorn et al., 2002). Accordingly, targeting stromal changes is gaining acceptance as an alternative option for treating cancer. For example, vascular endothelial growth factor (VEGF) inhibitors such as bevacizumab combat cancer by targeting tumor angiogenesis (Ferrara, Hillan, Gerber, & Novotny, 2004). These examples of tumor and tumor microenvironment contributions to response underscore the complexity of individual tumors and highlight the need for an expanded view when considering therapeutic response of individuals to cancer therapies.

While patient profiling can improve patient outcome, cancer mortality remains high and it is clear that we need additional approaches (Hidalgo et al., 2011). In response, there is once again increasing interest in integrating patient samples into *in vitro* models to predict *in vivo* response. These models, most commonly referred to as chemosensitivity and resistance assays (CSRAs), have the potential to provide a rapid, high throughput and inexpensive approach to predicting therapy for individuals, but have thus far faced challenges that have hindered their success (Bartlett, et al., 2014). Poor *in vitro* culture conditions (Bartlett, et al., 2014; Samson, Seidenfeld, Ziegler, & Aronson, 2004), the limited information offered by traditional *in vitro* readouts (Burstein et al., 2011; Wilmes et al., 2013), and tumor tissue heterogeneity (Bartlett, et al., 2014; Burstein, et al., 2011; Gerlinger, et al., 2012; Samson, et al., 2004) have been invoked to explain discordance between *in vivo* and *in vitro* therapeutic response.

In 2004 and 2011 the American Society of Clinical Oncology reviewed personalized *in vitro* models or what they referred to as “chemotherapy sensitivity and resistance assays” (CSRAs). In both instances, they recommended not to use these models to identify appropriate therapeutic agents outside of clinical trials but stressed their potential importance (Burstein, et al., 2011; Schrag et al., 2004). Advances in molecular biology, toxicology, biomedical/tissue engineering and other fields offer advances that may help move CSRAs into clinical use.

In an effort to improve CSRAs, we propose a guiding framework coined a therapeutic outcome pathway (TOP), to select culture conditions, cellular readouts and key components to include in a CSRA. The TOP concept is based on the adverse outcome pathway (AOP) framework used in toxicology to map the molecular, cellular and tissue level targets of a toxin or in this case a therapeutic. We will begin our discussion by addressing challenges with *in vitro* personalized models and the emerging solutions. This will familiarize the reader with the advantages of personalized *in vitro* models and the obstacles that hamper their success. We will then introduce the TOP framework and describe how it might be used to overcome these obstacles and maximize the information retrieved from a patient sample. The TOP framework will facilitate the development of personalized *in vitro* models because it will highlight what should be incorporated into models and what readouts will be most predictive of response. To illustrate the usefulness of the framework, we will provide an example of estrogen receptor (ER) positive breast cancer and tamoxifen treatment.

2. The nature of patient derived samples: Heterogeneity, sample acquisition, and challenges with primary cell culture

Intertumor heterogeneity (heterogeneity between tumors of different patients) has driven the need for personalized medicine (Burrell, McGranahan, Bartek, & Swanton, 2013). Even within the same cancer subtype, each patient's tumor can have distinctly different gene expression profiles, tumor microenvironments and behaviors (Ogino, Fuchs, & Giovannucci, 2012). Early attempts to predict *in vivo* response made use of immortalized cell lines *in vitro*. However, immortalized cell lines are highly selected subpopulations and do not adequately reflect the heterogeneous function and behavior of tumors (Cree, Glaysher, & Harvey, 2010; Dairkee et al., 2004). Consequently, there is increased interest in using primary cells in personalized *in vitro* models for evaluating drug response (Gerlinger, et al., 2012; Liotta & Petricoin, 2000; Longo, 2012).

While ideal, patient derived primary cells present their own set of challenges in areas such as sample acquisition, variability and culture. First, they are difficult to obtain in the standard diagnostic workflow. Traditionally, standard of care dictates that tissue removed during biopsy be immediately sent for pathological examination where it is assessed for morphologic and molecular markers that are indicative of disease stage and cancer subtype (Ivshina et al., 2006). Pathological review is well established and is a reliable method to gain valuable information about a patient's cancer, but it offers limited predicted power (Gravendeel et al., 2009; Hirsch et al., 2008; Sequist et al., 2011). Integrating patient samples into *in vitro* models requires that some part of the sample, which would ordinarily go to pathology, be relinquished for research purposes. With early detection and small tumor samples, obtaining a sample for research prior to or concurrent with pathological review is often not possible. Second, there are logistical complexities in transferring sample from operating room, to surgical pathology, and ultimately to researchers while maintaining cellular viability. Institutional tissue biobank can be useful in this regard (Bevilacqua et al., 2010; Roden et al., 2008). Once researchers have received primary samples, those tumor pieces are further processed, introducing additional variability.

Intratumor heterogeneity (heterogeneity within the same person's tumor) underlies many of the failures of CSRAs to date. The source of intratumoral heterogeneity stems from natural variations in tissue architecture, tumor evolution, selection of cells during processing, as well as changes induced when tumor cells are removed from the *in vivo* environment. This heterogeneity is thought to partly explain the high variability and response when utilizing primary cells (McAlpine, Eisenkop, & Spirtos, 2008). Perhaps this shouldn't be surprising since treatment induced regression *in vivo* can also sometimes be seen in some areas of an individual tumor but not others, indicating that a tumor sample may not be representative of overall tumor behavior (Gerlinger, et al., 2012).

Finally, primary cells become senescent after only a few passages and in many cases only a small number of patient cells are available for testing (Mitra, Mishra, & Li, 2013; Young et al., 2012). The limited availability and life span of primary cells makes it difficult to develop, optimize and validate new assay platforms. It is important, therefore, to consider if the information gained by personalized *in vitro* models outweighs what is gained from

evaluation of standard clinical parameters (Cree, et al., 2010). In summary, patient sample acquisition, variability and culture has hindered progress in personalized *in vitro* models.

3. The current state of primary cell based *in vitro* models: What has been done and how we can improve

The idea of personalized *in vitro* models is not new; researchers have been attempting to develop *in vitro* methods to predict therapeutic response for several decades (Bertelsen, Sondak, Mann, Korn, & Kern, 1984; Salmon et al., 1978). Despite these efforts, CSRAs are not recommended outside of a clinical trial setting (Burstein, et al., 2011). While these assays have had some success with predicting drug resistance, they generally fail to predict drug sensitivity (Fruehauf & Alberts, 2005). Overall, current personalized *in vitro* models do not offer significant clinical benefit and must be improved (Fruehauf, 2002; Fruehauf & Alberts, 2005; Wu, Zhu, Zhang, Shen, & Zhang, 2008). Several reviews evaluate the limitations of predictive *in vitro* models and their use in clinical practice (Ferriss & Rice, 2010; Fruehauf, 2002; Samson, et al., 2004).

Several potential contributing factors likely underlie the historical failures of CSRAs, but one significant problem is that *in vitro* models are too simplistic to accurately model and predict *in vivo* response to therapeutics (Bartlett, et al., 2014; Burstein, et al., 2011). In hope of developing more robust cancer models, some groups have begun to incorporate additional microenvironment components factors into *in vitro* cancer models (J. B. Kim, Stein, & O'Hare, 2004; Nyga, Cheema, & Loizidou, 2011; Shamir & Ewald, 2014; Weaver et al., 1997). In addition to creating more biologically relevant culture conditions, we speculate that analyzing several orthogonal readouts per sample will improve model predictability. Here, we will summarize the technical evolution of CSRA platforms. We will first discuss what these models have traditionally entailed and then discuss if incorporating additional microenvironmental factors and readouts into these platforms is needed to predict patient response.

3.1 Traditional CSRA platforms

In traditional *in vitro* CSRAs, researchers isolate cancer cells from the patient tumor, culture them on a two-dimensional (2D; flat plastic or glass surface) well plate and expose them to an array of drugs to assess response. These platforms typically assess response using one readout per sample, most often cell survival/death (Samson, et al., 2004). For example, the adenosine triphosphate (ATP), 3-(4,5-dimethylthiazolyl-2)-2, 5-diphenyltetrazolium bromide (MTT), extreme drug resistance (EDR), and ChemoFX assay have been widely used as the sole endpoint in personalized *in vitro* models (Cree & Andreotti, 1997). Several groups have attempted to use the ATP assay to predict drug effectiveness of patient derived cancer cells. However, the assay has been found to be sensitive but not specific, and could therefore predict drug sensitivity but not resistance (Sevin et al., 1988). One study reported increased overall survival in the ATP assay directed group (Ugurel et al., 2006) while another group reported that survival was not improved (Cree, Kurbacher, Lamont, Hindley, & Love, 2007). The MTT assay was also used to evaluate epirubicin and docetaxel sensitivity in primary human breast cancer cells and correctly predicted changes in cancer cell proliferation but not

overall tumor size (Singer et al., 2013). The MTT assay was used to predict drug effectiveness in 353 gastric cancer patients but no difference in clinical outcome was found between the control group and the MTT sensitive group (Wu, et al., 2008). EDR assays have also had mixed success, where some groups found that the test could predict patient survival where others could not (Joo et al., 2009; Matsuo, Eno, Im, Rosenshein, & Sood, 2010; Mehta et al., 2001). The ChemoFX assay is promising but may not be generalizable. Several groups have reported that the ChemoFX assay is predictive of patient response (Gallion et al., 2006; Mi et al., 2008) and that ChemoFX directed drug treatment improves overall survival (Herzog, Krivak, Fader, & Coleman, 2010). However, one group found that ChemoFX did not correlate with patient outcome and did not improve overall survival (Bhagwandin, Naffouje, & Salti, 2015). In the latter, the authors assessed tumors from patients with a variety of cancers, where ChemoFX has been mostly used to predict therapeutic response in ovarian cancer patients (Richard, Wells, Connor, & Price, 2015). Thus, there has been mixed success with the one readout, one cell type on a 2D architecture approach.

These models do not incorporate key features of the *in vivo* environment such as tissue structure, matrix composition, an immune component or stromal cells despite evidence suggesting the pivotal role each play in cancer progression (Allinen et al., 2004; Place, Jin Huh, & Polyak, 2011; T. N. Wang, Albo, & Tuszynski, 2002). Mounting evidence supports that the absence of these components result in a decreased drug resistance, suggesting incorporation of at least some of these factors is necessary to accurately predict drug response (Chitcholtan, Asselin, Parent, Sykes, & Evans, 2013; Gajewski, Schreiber, & Fu, 2013; Loessner et al., 2010; Majety, Pradel, Gies, & Ries, 2015; Pan et al., 2015; Z. Xu et al., 2013).

Cells cultured in 2D environments undergo changes in morphology, deposition of ECM proteins, migration, cell growth, invasion and apoptosis (Birgersdotter, Sandberg, & Ernberg, 2005; Chen et al., 2012; Justice, Badr, & Felder, 2009; Pampaloni, Reynaud, & Stelzer, 2007; Z. Xu, et al., 2013). Primary cells also lose critical cell functions under these conditions (Markovina et al., 2008; McCallum & Lowther, 1996). It has been shown that cells cultured in 2D conditions experience a reduction or complete loss of some receptors and signaling molecules known to be critical to tumor progression in many different cancers, such as androgen receptor, human epidermal growth factor receptor, and estrogen receptor (Hayward et al., 1995; Novaro, Roskelley, & Bissell, 2003; Pickl & Ries, 2008).

3.2 Emerging CSRA platforms with ECM and microenvironmental components

Researchers have attempted to improve predictability of *in vitro* models and have begun to develop more complex CSRAs that promise to be more robust by incorporating key features of the *in vivo* environment (Shamir & Ewald, 2014). We distinguish examples of predictive *in vitro* cultures that have incorporated primary cancer cells embedded in extracellular matrix proteins, from those with spheroid structure, organoid structure and/or incorporated stromal cell types into different categories (Fig. 1). These platforms can be well-plate based or constructed using microfluidic technologies. Microscale *in vitro* models can provide unique functionality and controllability (e.g., enhanced spatial and temporal controls) and

are emerging as practical tools to investigate tumor-stroma interactions in various three dimensional (3D) conditions more efficiently. The fundamentals and applications of microfluidic *in vitro* models have been extensively reviewed (Khademhosseini, Langer, Borenstein, & Vacanti, 2006; Morales et al., 1995; Sackmann, Fulton, & Beebe, 2014; Young & Beebe, 2010). One well-known advantage of microfluidic systems is the reduced number of cells required for each endpoint, which makes the microfluidic system especially attractive when working with patient samples (Young, et al., 2012).

3D cell culture models allow cells to be cultured in natural hydrogels, such as collagen or Matrigel, or synthetic biomaterials, such as polyethylene glycol (PEG) gels. The incorporation of extracellular matrix (ECM) components into cultures has shown striking differences between 3D and 2D cultures, including differences in morphology, adhesion, polarity, differentiation and gene expression (Kenny et al., 2007; Pampaloni, et al., 2007; Sung et al., 2013; Yamada & Cukierman, 2007). For instance, in some cases culturing primary cells in 3D conditions can prevent the prototypical loss of receptor expression seen when primary cells are placed in 2D culture conditions (Novaro, et al., 2003; Yang et al., 2000). Morphological differences have also been reported in primary cells when cultured in 2D vs. 3D, where a more *in vivo* like morphology was observed in 3D (Z. Xu, et al., 2013). It is speculated that these differences are because materials used in 3D cultures provide structural and chemical components that are similar to ECM found *in vivo* (Wolf et al., 2009). It is also important to note that chemical and mechanical properties of various ECM materials influence cell behaviors in 3D *in vitro* cultures considerably (Loessner, et al., 2010; Weaver, et al., 1997). Various synthetic and natural materials that have been used in designing 3D *in vitro* system have been previously reviewed (Nyga, et al., 2011; Tibbitt et al., 2015; X. Xu, Farach-Carson, & Jia, 2014). The collagen gel droplet embedded culture drug test (CD-DST) is one example of a 3D CSRA. Similar to 2D predictive assays, the predictive value of the CD-DST is mixed because in some cases it has high sensitivity but low specificity (Higashiyama et al., 2010; Takebayashi et al., 2013).

Co-culture of cancer cells with other cellular components of the microenvironment has been shown to influence the growth rate, protein secretion and drug sensitivity of cancer cells (Sadlonova et al., 2005). Several groups incorporated patient stromal cells into a variety of personalized *in vitro* platform architectures (K. U. Kim et al., 2005; Pak et al., 2015; Ruppen et al., 2015; Z. Xu, et al., 2013; Young, et al., 2012). In four cases that assessed the influence of stromal cells on drug sensitivity, the authors found differences in the effective dose of primary cancer cells cultured in a monoculture compared to co-culture, highlighting the importance of integrating supportive cell types into predictive models (Pak, et al., 2015; Ruppen, et al., 2015; Z. Xu, et al., 2013; Young, et al., 2012). The majority of these platforms assessed cytotoxicity as their readout (Hsieh, Chen, Huang, Wang, & Wu, 2015; Z. Xu, et al., 2013), however, a microscale platform developed by Young et. al allowed a functional analysis of patient samples in addition to evaluation of cell viability, where NF-kb and STAT3 nuclear translocation were evaluated following drug treatment (Young, et al., 2012).

Spheroid culture models, where cells self-arrange or proliferate into spherical balls of cells, sometimes with a hollow lumen, are another popular tool to study cancer in a more

physiologically relevant system (Desoize, Gimonet, & Jardiller, 1998). There is evidence that organization of cells in spheroids, rather than a monolayer, better reflects the *in vivo* behavior of tumors (Hirschhaeuser et al., 2010; Shield, Ackland, Ahmed, & Rice, 2009; Weiswald, Bellet, & Dangles-Marie, 2015). While there are several techniques to generate spheroids, most commonly cells are placed in a 2D or 3D environment that encourages cell-cell adhesion rather than matrix adhesion (Santini, Rainaldi, & Indovina, 1999). With growing recognition of the importance of 3D microenvironment, spheroids also can be generated in various biomaterials to achieve spheroid-ECM interactions (Hsiao et al., 2009; Loessner, et al., 2010; Ong et al., 2010). Several groups have compared response when primary tumor cells are cultured in traditional 2D conditions and tumor spheroids. One group cultured primary oral cancer cavity cells in microscale 2D, 3D or spheroid platform and found that the culture method influenced drug sensitivity, metabolic activity and proliferation (Hsieh, et al., 2015). Kim et al. found that patient derived tumor spheroids composed of mesothelioma cells, macrophages and collagen demonstrated apoptotic resistance to chemotherapeutics, whereas mesothelioma cell monolayers did not (K. U. Kim, et al., 2005). Other groups have used spheroids to evaluate sensitivity to chemotherapeutics, where the authors suggested that the *in vitro* model could be used to determine the dose most appropriate for a given patient. However, this claim was not validated and purely speculative; these authors did not (to our knowledge) use doses identified in the *in vitro* assay to guide the treatment of patients in the clinic (Kondo et al., 2011).

Finally, the emergence of patient-derived organoids has garnered a great deal of interest in the personalized medicine field. Organoids are derived from stem cells and are considered more complex/differentiated than spheroids because they recapitulate some aspects of organ structure and function including differentiation into multiple cell types (i.e. basal and luminal epithelial cells) (Mustata et al., 2013; Sachs & Clevers, 2014; Sasai, Eiraku, & Suga, 2012). Perhaps the most discussed example is the generation of crypt-villus structures from a single intestinal stem cell (Sato et al., 2009). Patient-derived organoids are advantageous because they can be cultured long term, are amenable to drug screening and retain histological, immunohistochemical and genetic features of the tissue they were derived from (Gao et al., 2014; Huang et al., 2015; Sato et al., 2011; van de Wetering et al., 2015). One study sequenced the genomes of organoids derived from castration resistant prostate cancer patients to identify molecular signatures of drug resistance. They also evaluated the influence of three cancer drugs on the growth rate of the organoids and demonstrated that analyzing both drug sensitivity and genomic data can offer a more complete insight into patient response. (Gao, et al., 2014). Another study generated organoids from 20 colorectal carcinoma patients and evaluated 80 different compounds on organoid viability. The authors coupled genomic data with *in vitro* drug response to gain a more comprehensive understanding of how each patient may respond to a given drug. As expected, these studies revealed heterogeneity in patient response, for instance, organoids with KRAS mutations were more resistant to anti-EGFR inhibitors. Interestingly, these studies also identified patients who had high sensitivity to compounds with no known genetic biomarker. (van de Wetering, et al., 2015).

3.3 Choosing the right platform complexity, culture conditions and assay readouts

We argue that using more physiologically relevant cultures may improve the likelihood that an *in vitro* model can predict response, and should be further explored. Ideally, a model would incorporate all components of the tumor and microenvironment but there is a trade-off between complex and physiological relevance with reproducibility, ease of use, and cost. It is essential that we recapitulate the *in vivo* environment well enough so we can accurately model drug response, while also maintaining the simplicity necessary for evaluating drugs in a manner that is succinct and cost efficient (Loessner, et al., 2010). In section 4.0, we will offer our perspective on how to guide this process.

Some authors have argued that the simplicity of a “one readout approach” is beneficial for evaluating clinical response (Fruehauf, 2002). While this approach may allow a more straightforward assessment of *in vitro* response, cancer is a complex disease that occurs as a result of multiple hits or changes to several critical components (e.g. changes in gene expression, stromal changes, angiogenesis etc.) (Loeb, Loeb, & Anderson, 2003; Tuxhorn, et al., 2002). Moreover, some subtypes of cancers are tightly regulated by a biological pathway, such as the androgen receptor pathway in prostate cancer (M. H. Tan, Li, Xu, Melcher, & Yong, 2015) or the estrogen receptor pathway in breast cancer (Orlando et al., 2010). These pathways are complex and in order to determine if a drug can act on and produce a therapeutic response to combat a subtype of cancer, it is often necessary to run multiple assays both *in vitro* and *in vivo*. Using one endpoint per *in vitro* assay has been sufficient for drug development because it is possible to run several separate experiments on immortalized cell lines and use *in vivo* models to evaluate tissue level changes (Hoelder, Clarke, & Workman, 2012). However, this is problematic when working with patient samples because only a small number of cells are available for testing and this approach is costly and time consuming.

We hypothesize that one of the major challenges that has hindered the success of personalized *in vitro* models is the limited information elicited from each sample. To increase confidence that personalized *in vitro* models can predict drug response, it is important to analyze multiple readouts in a single sample at different levels of organ complexity. While we were previously limited by the available technologies, there has been a great deal of improvement in assay development, analytical equipment (e.g. microscopes, plate readers), sequencing and culture platform architectures (Gerets et al., 2009; Provenzano et al., 2006; Veer & Bernards, 2008; Wilmes, et al., 2013). Genomic characterization of tumors enables the identification of mutations, gene deletions, etc. that are indicative of drug sensitivity/resistance (Davies & Samuels, 2010). Additionally, reagents that readout diverse physiologies and are compatible with live cell assays have been developed, enabling researchers to multiplex several readouts in one model (Butterick et al., 2014; Hooper, 2011). The emergence of 3D, spheroid, and other organotypic cultures allows the analysis of tissue level changes (Bischel, Young, Mader, & Beebe, 2013; Jimenez-Torres, Peery, Sung, & Beebe, 2015; Zervantonakis et al., 2012). The term “3D model” has been used interchangeably with “organotypic model”. While similar, the organotypic model refers more specifically to 3D models that faithfully recapitulate *in vivo* like structures *in vitro*. In particular, the integration of microfluidics with organotypic models enables the generation of

tissue structures, such as a circular shaped lumen system, with enhanced controls and throughput (Bischel, et al., 2013). Organotypic models enable researchers to evaluate changes that were previously difficult to quantify *in vitro*, including key events in cancer progression, such as invasion, angiogenesis, intravasation, and extravasation (Bischel, Beebe, & Sung, 2015; Jeon et al., 2015; Jimenez-Torres, et al., 2015; Zervantonakis, et al., 2012). Evaluating responses from functional assays in addition to the molecular signatures of a given tumor would offer valuable insight into optimal drug options for the individual patient.

The development of models that analyze multiple endpoints at a molecular, cellular and tissue level may increase the likelihood that evaluating a patient sample will provide predictive information for identifying successful treatments. However, with the countless assays available and many changes that occur during cancer, it is often difficult to determine which endpoints would provide the most valuable information for evaluating drug response.

4. The therapeutic outcome pathway: A conceptual framework to aide in the development and application of personalized CSRAs

We have so far discussed how increasing the physiological relevance of our cultures and integrating readouts at a molecular, cellular and tissue level should improve the predictive value of personalized *in vitro* models. While these advantages may be intuitive, it is less apparent what specific elements of the *in vivo* environment should be incorporated into models to best evaluate response for a given therapeutic. Cancer development and progression can vary drastically depending on cancer subtype (Sikora et al., 1999). Even therapeutics that target the same cancer subtype can work through different mechanisms and have different risk factors for resistance (Gottesman, 2002; Normanno et al., 2005). Consequently, factors that are important when evaluating drug response may vary depending on the cancer subtype and therapeutic being tested. To determine what factors are most important when assessing therapeutic response, we must ask several questions. For instance, 1) are there any microenvironmental components that are important in disease progression or are targeted by the therapeutic? 2) What events at a cellular, molecular and tissue level are most important when evaluating response to a given cancer drug? 3) Are there any genetic signatures that might predispose a patient to therapeutic resistance or toxicity? To better organize this information, we propose the use of the therapeutic outcome pathway (TOP), a variation of the adverse outcome pathway (AOP), a framework commonly used to model mechanisms of drug/chemical induced toxicities (Vinken, 2013). In context of personalized medicine, the purpose of the TOP is to identify what cellular and microenvironmental factors are essential and what endpoints are critical for evaluating response to a given therapeutic, in order to maximize the likelihood that a personalized *in vitro* model is able to predict therapeutic response.

4.1 Introduction to Outcome Pathways

It is our perspective that a generalizable framework that graphically describes the essential components and molecular, cellular and tissue level events that occur during cancer progression and targeted therapy is key to advancing personalized CSRAs. The AOP

framework, utilized in designing toxicity screening platforms, is an existing framework that has already been used to predictively model disease and screen for compounds that speed cancer progression (Edwards, Tan, Villeneuve, Meek, & McQueen, 2016; Vinken, 2013). The visual representation of key events in disease progression provides a clear understanding of how a chemical exposure can result in an adverse outcome. AOPs employ a hierarchical approach of increasing biological complexity from the earliest molecular event (molecular initiating event) to the advanced tissue level adverse outcome (tumor growth) and beyond to death (Fig. 2) (Vinken, 2013). Some examples include estrogen receptor driven breast cancer, androgen receptor driven prostate cancer or NFkB activation driven multiple myeloma (Markovina, et al., 2008; Russo & Russo, 2006; M. E. Tan, Li, Xu, Melcher, & Yong, 2014). The intermediate steps or key events that directly link the molecular initiating event and adverse outcome are mapped using the basic science literature and noting the weight of evidence that connects these events. Key events include responses that are both driven by the molecular initiating event but occur independently of each other perhaps even in different cell types or tissues, referred to as orthogonal events. Previous reports have discussed strategies to construct AOPs, including a formal document published by the Organisation for Economic Co-operation and Development (OECD) (Ankley et al., 2010; OECD, 2013; Vinken, 2013). While AOPs are constructed for the purpose of identifying early molecular or cellular endpoints to incorporate into chemical screening platforms, TOPs go beyond this utility to incorporate molecular, cellular and tissue level effects.

4.2 Identifying key components and readouts for CSRAs

We propose that the outcome pathway framework could be used to improve predictive medicine by guiding the development and implementation of personalized CSRA platforms. In this regard, the AOP would be used in the identification of key components of a system that are needed to recapitulate the mechanism by which a given compound causes toxicity (the adverse outcome, cancer progression and death). The identification of events at different levels of biological organization is especially useful when developing organotypic models.

In addition to identifying microenvironmental components that are needed to recapitulate a therapeutic effect, we have found that an extension of the AOP, what we have coined the therapeutic outcome pathway (TOP), can help identify how proposed therapeutics target key events of an AOP (e.g. which event(s) of the AOP does the therapeutic target). The TOP will help pinpoint key events that may be useful as readouts to determine therapeutic effectiveness. In contrast to the traditional AOP framework, a TOP should also include key effectors that alter the metabolism and excretion of the therapeutic as well as measures of therapeutic resistance, as these are all critical in therapeutic response and effectiveness.

As an example, we created an AOP for a specific cancer subtype, estrogen receptor driven breast cancer, which is shown below in section 4.3. We then developed a TOP in section 4.4 by assessing tamoxifen in context of the AOP. We have summarized how the TOP can guide the development and implementation of personalized *in vitro* models in figure 3.

4.3 Utilizing the AOP of estrogen receptor activation and breast cancer progression to guide the development of CSRAs

Identifying the key events that occur during ER+ breast cancer progression can help determine what microenvironmental components and readouts (readouts are discussed in the next section) may be most predictive when modeling breast cancer response. We have developed the ER mediated breast cancer AOP to frame this information and help make decisions regarding model components.

Major sources of estrogen receptor (ER) agonists include estrogen synthesis in the ovaries and local synthesis through conversion of androgens to estrogens via aromatase (Mobley & Brueggemeier, 2004; Simpson, 2003; Williams & Phillips, 2000). Estrogens and other ER agonists bind to ER and stimulate ER activation, the molecular initiating event of the AOP (McDonnell & Norris, 2002). Initial cellular changes include ER-induced genomic and non-genomic effects. In the genomic pathway, estrogen binding to ER results in a series of events (posttranslational modification, dimerization, conformational change). This leads to ER binding either directly (the classical pathway) (Demirpence, Duchesne, Badia, Gagne, & Pons, 1993; Doisneau-Sixou et al., 2003; Hayashi et al., 2003) or indirectly (the non-classical pathway) (Hall, Couse, & Korach, 2001; McDonnell & Norris, 2002; Musgrove & Sutherland, 2009; O'Lone, Frith, Karlsson, & Hansen, 2004) to DNA and the initiation of transcription. The rapid, non-genomic effects of ER are less characterized but are thought to include stimulation of signaling pathways through mechanisms such as an increase in production of secondary messengers (Bjornstrom & Sjoberg, 2005; Improt-Brears et al., 1999).

Following the initial cellular responses, there are primary cellular responses such as changes in gene transcription and protein production (Bjornstrom & Sjoberg, 2005; Bourdeau et al., 2004; Doisneau-Sixou, et al., 2003; Hayashi, et al., 2003). Well-characterized ER induced cellular responses of breast cancer epithelial cells include changes in mitochondrial dynamics (Sastre-Serra, Nadal-Serrano, Pons, Roca, & Oliver, 2012; Sastre-Serra et al., 2015; Sastre-Serra et al., 2010), oxidative stress and DNA damage (Caldon, 2014; Cavalieri, Frenkel, Liehr, Rogan, & Roy, 2000; Mobley & Brueggemeier, 2004). These changes are thought to influence proliferation (Bjornstrom & Sjoberg, 2005; Ciocca & Fanelli, 1997; Dabrosin, Margetts, & Gauldie, 2003; Lee et al., 1999; Zivadinovic, Gametchu, & Watson, 2004) and apoptosis (Bailey, Shin, Westerling, Liu, & Brown, 2012; T. T. Wang & Phang, 1995; Yamamoto et al., 2014). For instance, ER induced changes in mitochondrial dynamics, oxidative stress, and DNA damage have been shown to increase proliferation of ER+ breast cancer epithelial cells (Caldon, 2014; Felty & Roy, 2005; Felty, Singh, & Roy, 2005). Modulation of mitochondrial dynamics, proliferation and oxidative stress increase DNA damage, which can lead to pro-cancerous mutations (Caldon, 2014; Mobley & Brueggemeier, 2004; Sastre-Serra, et al., 2010). Estradiol exposure can also result in DNA damage through estrogen metabolism, although this mechanism is independent of ER activation (Cavalieri, et al., 2000; Yager & Davidson, 2006). While more controversial, some authors have observed an ER induced increase in motility (Saji et al., 2005; Thompson et al., 1988; Zheng et al., 2011) where others have reported a decrease (Albini et al., 1986; Bracke et al., 1994; Platet, Cathiard, Gleizes, & Garcia, 2004).

Cells from the microenvironment can also be incorporated in this framework. Endothelial cells express ER and are stimulated by ER to proliferate and migrate (Dabrosin, Margetts, et al., 2003; Sengupta, Banerjee, Saxena, & Banerjee, 2003; Simoncini et al., 2004). Macrophages, fibroblasts and adipocytes undergo changes and become ‘cancer associated’ during breast cancer (Mao, Keller, Garfield, Shen, & Wang, 2013; Obeid, Nanda, Fu, & Olopade, 2013; J. Tan, Buache, Chenard, Dali-Youcef, & Rio, 2011; Yamaguchi, 2007). While these cells are influenced by and influence cancer progression, it is unclear if there is a direct relationship between estrogen receptor activation and cancer association, although some evidence supports a direct relationship between ER activation and macrophage function (Calippe et al., 2010; Campbell et al., 2014). M2 macrophages, coined tumor-associated macrophages (TAMs) secrete cytokines and other factors that stimulate angiogenesis, suppress immune response and encourage tumor cell proliferation and invasion (Bohrer & Schwertfeger, 2012; Mor et al., 1998). Cancer associated fibroblasts (CAFs) increase proliferation and invasion of cancerous epithelial cells and recruit inflammatory and endothelial cells to tumor sites (Aboussekhra, 2011; Soon et al., 2013). Cancer associated adipocytes (CAAs) become delipidated, obtain a fibroblast-like morphology and encourage cancer cell proliferation and invasion (Mao, et al., 2013; J. Tan, et al., 2011). CAFs, CAA and TAMs secrete proteins that degrade and remodel the extracellular matrix, such as matrix metalloproteinases (Dirat et al., 2011; Obeid, et al., 2013; Sounni & Noel, 2013). They also produce unusually high levels of aromatase that greatly contribute to estrogen rich environment found in ER+ breast cancer tissue (Bulun et al., 2009; Mor, et al., 1998; Santen et al., 1997; J. Tan, et al., 2011; van Landeghem, Poortman, Nabuurs, & Thijssen, 1985).

The next level examines tissue-level responses. Decreased apoptosis and proliferation cause ductal filling (tumor growth) (Marchese & Silva, 2012) and changes in endothelial cell migration and proliferation result in increased angiogenesis, which supports tumor growth (Applanat, Buteau-Lozano, Herve, & Corpet, 2008; Dabrosin, Margetts, et al., 2003; Dabrosin, Palmer, Muller, & Gauldie, 2003; Sengupta, et al., 2003). The extracellular matrix is remodeled due to stromal cell induced alterations of matrix stiffness, collagen I orientation (the most abundant protein in the breast), and the composition of other extracellular matrix proteins (Haslam & Woodward, 2003; Ioachim et al., 2002; Lu, Weaver, & Werb, 2012; Provenzano, et al., 2006; Provenzano et al., 2008). While important in disease progression, it is unclear if ECM changes are directly related to ER. Finally, these changes collectively encourage invasion out of the mammary duct and colonization of secondary sites, which is ultimately what leads to the adverse outcome, breast cancer related death (Provenzano, et al., 2006; X. H. Zhang, Giuliano, Trivedi, Schiff, & Osborne, 2013). Whether ER plays a direct role in motility and invasion processes has not been established, in part due to lack of good models of ER+ metastasis. Nevertheless, the existence of ER+ CTCs and metastatic tumors provide evidence that ER+ cancer cells are subject to the same pressures at the tissue level (Paoletti et al., 2015).

To ensure reliability and robustness of our AOP, we have used the Bradford Hill Criteria outlined by the OECD to assess weight of evidence for each key event (Table 1) (OECD, 2012). Here, we have identified several key events that link the molecular initiating event (ER activation) and the adverse outcome (breast cancer related deaths), and several

microenvironmental components that play a role in breast cancer progression. While it is not feasible to include all of these factors into a CSRA, we can use the framework depicted in Fig. 4 as a guide to determine what components are most important. The cancerous epithelial cells incorporated in the model should be ER+, as ER activation is thought to drive the AOP. ER status is commonly lost when primary cells are placed in 2D conditions. However, Novaro et. al reported that ER expression is better maintained in murine cells when cultured on an extracellular matrix (Novaro, et al., 2003). Therefore, ECM proteins such as laminin or collagen IV could be incorporated into the model to help maintain ER function. In addition to epithelial cells, endothelial cells may be of importance when modeling disease progression. Endothelial cells are directly influenced by ER activation and angiogenesis supports tumor growth and epithelial cell invasion. Fibroblasts could also be important to include into the CSRA. As discussed in section 3.2, fibroblasts influence drug sensitivity. In context of the AOP, fibroblasts play a role in extracellular matrix reorganization, epithelial proliferation and provide routes of invasion.

4.4 Utilizing the TOP of tamoxifen and ER mediated breast cancer progression to help identify predictive readouts

Tamoxifen is a selective estrogen receptor modulator that is commonly used to combat ER+ breast cancer progression (Osborne & Schiff, 2011). Unfortunately, therapeutic response to tamoxifen can vary and it is often unclear if a patient will benefit from the drug (Musgrove & Sutherland, 2009; Osborne & Schiff, 2011; Roop & Ma, 2012). The application of tamoxifen towards the ER mediated breast cancer progression AOP can help frame what readouts are most important for predicting tamoxifen effectiveness. This will generate the tamoxifen TOP. Given the limited success of CSRAs to date, we will outline considerations relevant to inclusion of patients within a study, as well as factors that influence choice of endpoints to monitor.

It is important to first discern what patient factors may result in a CSRA failing to predict response including ADME and resistance mechanisms. Tamoxifen is a prodrug and is metabolized to more active forms, such as 4-hydroxytamoxifen and endoxifen. These compounds are much more potent at modulating ER driven genes than tamoxifen because they have a higher affinity for ER. Therefore, the effectiveness of tamoxifen as a therapeutic agent can be strongly influenced by polymorphisms of genes that encode for tamoxifen metabolizing enzymes. Several cytochromes P450 (CYP) are responsible for tamoxifen metabolism including CYP2D6, CYP3A4, CYP3A5, CYP2B6 and CYP2C19 (Desta, Ward, Soukhova, & Flockhart, 2004). Individuals who carry alleles for these enzymes that exhibit reduced enzymatic activity, “poor metabolizers”, can less efficiently generate more active compound and thus, experience less therapeutic benefits of the drug (Hayes & Lewis-Wambi, 2015). CYP2D6 is primarily responsible for metabolizing tamoxifen to endoxifen. Poor metabolizers of CYP2D6 gene have lower concentrations of endoxifen, and a worse clinical outcome (Beverage, Sissung, Sion, Danesi, & Figg, 2007; Lim et al., 2011; Y. Xu et al., 2008). Enzymes that are responsible for the elimination of tamoxifen, such as sulfotransferase 1A1 and UDP-glucuronosyltransferases 2B7 and 1A8 may also play a role (Blevins-Primeau et al., 2009; Gjerde et al., 2008; Lazarus, Blevins-Primeau, Zheng, & Sun, 2009). Similarly, genetic polymorphisms that encode for drug transporters may also

influence clinical efficacy. P-glycoprotein (ABCB1) is involved in the transport of tamoxifen metabolites and differences in ABCB1 levels are thought to influence serum concentrations of these metabolites (Cronin-Fenton, Damkier, & Lash, 2014; Iusuf et al., 2011; Kiyotani, Mushiroda, Nakamura, & Zembutsu, 2012).

Combining tumor genome profiles with CSRA could enhance their predictive power. Tumors can express predictive therapeutic resistance markers within their gene expression profile (Massarweh & Schiff, 2006; Orlando, et al., 2010). For instance, breast cancer cells that express high levels of the ER co-activator, amplified in breast cancer 1 (AIB1) and nuclear receptor HER2, are resistant to tamoxifen inhibition and instead stimulated by tamoxifen. For individuals with this tumor profile, tamoxifen is not recommended as therapy because they are very prone to resistance (Shou et al., 2004). Therefore, incorporating the genomic and pathological data could help stratify patient samples and refine the design and analysis of the CSRA.

Given that the patient is a good candidate for a therapeutic, it is helpful to outline the key portions of the AOP targeted by the therapy to determine the best readouts for predicting therapeutic response (TOP depicted in Fig. 5, where targeted key events are highlighted in pink). In the case of tamoxifen and its metabolites, they compete with estrogen for ER binding and modulate ER induced genomic effects through the classical pathway. Consequently, tamoxifen influences ER driven gene transcription and protein production (Hayes & Lewis-Wambi, 2015; Webb, Lopez, Uht, & Kushner, 1995). Through modulating the expression of ER-targeted genes, tamoxifen decreases proliferation and increases apoptosis (Ciocca & Fanelli, 1997; Ellis et al., 1997). There are conflicting reports on the ability of tamoxifen to modulate cell motility and invasion both *in vitro* and *in vivo*, with some reports claiming a protective effect while others claim an invasive effect (Kuss, Muss, Hoen, & Case, 1997; Matsuoka et al., 2009; G. J. Zhang et al., 1999). However, clinical trials have shown that tamoxifen decreases the risk of invasive breast cancer and reduces breast cancer related deaths due to its ability to reduce metastasis (Fisher et al., 1998; Group, 1998). Tamoxifen decreases endothelial cell proliferation and migration, thereby decreasing angiogenesis (McNamara et al., 2001). While it is clear that tamoxifen has anti-angiogenic effects, there is speculation whether this is independent of ER (Blackwell et al., 2000).

TOPs can help us identify what readouts on a molecular, cellular and tissue level will be most useful for the CSRA. While it is not possible to measure all key events that tamoxifen targets, the TOP at least highlights which may be considered. In this case, evaluating the ability of tamoxifen to influence gene and protein expression, invasion and motility through ECM and in the context of multiple cell types, and determination of tamoxifen metabolism, provides a more comprehensive analysis that adds information to proliferation and apoptosis that could better inform patient response to tamoxifen and provide insight into complementary strategies.

5. Concluding Remarks

The application of AOP/TOPs towards personalized medicine has great potential because it helps organize information about a given therapeutics mechanism of action within its

cellular context. While the AOP has been criticized for simplifying a complex process (e.g. breast cancer development) (Vinken, 2013), providing a clear representation of how a therapy impacts outcome could reveal key events or properties of the system that improve response prediction and hence, aide in the decision-making process. The example shown above helps illustrate how the AOP/TOP framework can be used to choose components and identify readouts when building CSRA's using a well-characterized anti-cancer agent (overall process depicted in Fig. 3). The general AOP framework highlights what role cell types and other microenvironmental components play in progression. Understanding these components can help determine what needs to be incorporated into *in vitro* models to reflect *in vivo* response. The TOP framework can help identify what readouts are most important when predicting drug effectiveness. We believe that in addition to aiding in the development of CSRA's, the TOP can also be used to improve the predictive potential of other types of CSRA platforms, such as whole mount tissue slices or tissue scaffolds. We are currently applying these concepts to observe therapeutic responses to neoadjuvant chemohormonal therapy in a clinical trial for advanced prostate cancer patients. We will utilize the TOP-based approach to identify readouts and the microenvironmental compartments that are most important to examine when evaluating therapeutic response in PSMA-PET guided responding and resistant tumor foci *ex vivo*. While CSRA's are not widely used, we believe that the TOP/AOP can help overcome some of the problems hindering their success.

Acknowledgments

This review was supported by EPA grant number 83573701, University of Wisconsin Carbone Cancer Center, Grant P30 CA014520, NIH T32 CA157322, NIH R01CA186134 and Prostate Cancer Foundation Challenge Award.

Abbreviations

AOP	adverse outcome pathway
ATP	adenosine triphosphate
CAF	carcinoma associated fibroblast
CSRA	chemotherapy sensitivity and resistance assays
CYP	cytochrome P450
ECM	extracellular matrix
ER	estrogen receptor
ERE	estrogen response element
MTT	3-(4,5-dimethylthiazolyl-2)-2, 5-diphenyltetrazolium bromide
2D	two dimensional
3D	three dimensional
OECD	Occupational Economic Cooperation Development

TOP therapeutic outcome pathway

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Culture Method	Schematic	Architecture	Example
Traditional (2D)		Monolayer of cancer cells on a flat plastic or glass surface	Sevin et al., 1998; Singer et al., 2013; Wu et al., 2003 Mehta et al., 2001 Matsuo et al., 2010 Hsieh et al., 2015 Kim et al., 2015 Pak et al 2015 Edmond et al, 2012
3D		Cancer cells embedded in matrices such as collagen I or Matrigel	Xu et al., 2013 Higashiyama et al., 2010 Takebayashi et al., 2013 Kim et al., 2005 Hsieh et al., 2015
Coculture		Cancer cell type along with another cell type of the microenvironment (fibroblast, non cancerous epithelial cell, etc.)	Xu et al., 2013 Janine et al., 2015 Edmond et al., 2012 Kim et al., 2005, Pak et al., 2015
Spheroid		Cell aggregates in a 3D or 2D environment that prevents cell-matrix adhesion and stimulates cell-cell adhesion	Kim et al., 2005 Konodo et al., 2015 Hsieh et al., 2015 Xu et al., 2013 Janine et al., 2015
Organoid		Organ-like structure typically derived from a single stem cell	Gao et al., 2014 van de Wetering et al., 2015 Huang et al., 2015

Figure 1. Examples of traditional and emerging personalized *in vitro* platforms

While personalized *in vitro* platforms have traditionally evaluated cancer by studying one cell type on a plastic or glass surface, researchers have begun to increase physiological relevance by incorporate extracellular matrix proteins, additional cell types and/or structure.

Figure 2. The AOP framework

A typical layout of an AOP, a framework that defines the key events between a molecular initiating event and an adverse outcome.

Figure 3. Utilizing therapeutic outcome pathways in predictive medicine

The AOP/TOP framework is used to identify the key microenvironment components and assay readouts/endpoints that are critical to model therapeutic response and resistance. TOPs frame the key events that a therapeutic targets and know resistance mechanisms, thus highlighting what readouts at a molecular, cellular and tissue level will be most useful when predicting response and resistance. The information gathered *in vitro* can be used to predict patient response.

Figure 4. The adverse outcome pathway of ER mediated breast cancer progression

We have identified the key events that occur between the molecular initiating event, estrogen receptor activation and the adverse outcome, breast cancer related death. This framework highlights what microenvironmental factors may be important when evaluating response.

Figure 5. The therapeutic outcome pathway of tamoxifen and ER mediated breast cancer progression

We have identified the key events of the AOP that tamoxifen targets in pink. The TOP emphasizes what events are important when evaluating tamoxifen effectiveness. This map can help determine what readouts should be incorporated into CSRAs. *Evaluation of gene expression should include known markers of tamoxifen resistance, in addition to tamoxifen-targeted genes.

Table 1

A weight of evidence assessment was done for each key event according to the Bradford Hill Criteria.

<i>Event</i>	<i>Weight of Evidence</i>
<i>Molecular Initiating Event</i>	
Estrogen receptor activation	Very Strong
<i>Cellular Effects (Initial)</i>	
ER binding to DNA (classical)	Very Strong
ER binding to T.F. to DNA (non classical)	Very Strong
Second messenger production	Strong
Activation of signaling pathways	Strong
<i>Cellular Effects (Primary)</i>	
Gene expression	Very Strong
Protein production	Very Strong
<i>Cellular Effects (Secondary)</i>	
Proliferation	Very Strong
Apoptosis	Very Strong
Oxidative Stress	Very Strong
DNA Damage	Very Strong
Mitochondrial Activity	Very Strong
Motility	Moderate
<i>Cellular Effects (Stromal)</i>	
Endothelial Proliferation + Migration	Very Strong
Cancer association of fibroblasts	Weak
Cancer association of adipocytes	Weak
Cancer association of macrophages	Moderate
<i>Tissue Effects</i>	
Angiogenesis	Very Strong
ECM Composition	Moderate
Tumor Growth	Very Strong
Invasion	Moderate