Melanoma Models for the Next Generation of Therapies

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Summary

There's a lack of appropriate melanoma models that can be used to evaluate the efficacy of novel therapeutic modalities. Here, we discuss the current state of the art of melanoma models including genetically engineered mouse (GEM), patient-derived xenograft (PDX), zebrafish and *ex vivo* and *in vitro* models. We also identify five major challenges that can be addressed using such models, including metastasis and tumor dormancy, drug-resistance, the melanoma immune response, and the impact of aging and environmental exposures on melanoma progression and drug resistance. Additionally, we discuss the opportunity for building models for rare subtypes of melanomas, which represent an unmet critical need. Finally, we identify key recommendations for melanoma models that may improve accuracy of pre-clinical testing and predict efficacy in clinical trials, to help usher in the next generation of melanoma therapies.

Introduction

Melanoma constitutes a prime example of how basic and translational research have improved the prognosis for cancer. We have made remarkable progress in the last 10 years, with 13 new melanoma therapies approved in the US, including both targeted and immune-based therapies (**Fig. 1**) (Luke et al., 2017). Standard of care includes treatment with immune checkpoint inhibitors (ICI), either alone or in combination, as well as targeted inhibitors of $BRAF^{V600E}$ and MEK kinases in the mitogen-activated protein kinase (MAPK) pathway. Interleukin 2 (IL-2), oncolytic viral, and interferon therapy remain options for a subset of patients.

These treatments have led to tremendous improvements in the survival and quality of life for advanced patients, who before the modern era could expect a 5-year survival rate of ~10% (Balch et al., 2001). For instance, an objective response rate of 61% was seen in patients treated with combination ipilimumab (anti-CTLA-4) and nivolumab (anti-PD-1) (Postow et al., 2015). In a separate study, 52% of patients receiving this combination were alive at 5 years compared to 26% treated with ipilimumab alone (Larkin et al., 2019). Approvals of targeted and immunotherapies for patients with Stage III operable melanomas (adjuvant therapy), have further extended the number of patients who can benefit from these therapeutic advances (Eggermont et al., 2018; Long et al., 2017; Robert et al., 2019; Weber et al., 2017). Likely reflecting these advances, deaths from melanoma dropped by an unprecedented 18% from 2013-2016 among people of European ethnic background, the group most affected by melanoma, which is the largest drop seen over such a short period for any type of cancer (Berk-Krauss et al., 2020).

Despite this progress, current therapies are not a cure for most patients with metastatic melanoma, which is a genetically heterogeneous disease, particularly in ~50% of patients whose tumors lack a BRAF mutation, and in patients with rare melanoma

subtypes (e.g., uveal, acral, mucosal). Many patients are inherently resistant to these therapies (primary resistance), or melanomas recur and become resistant following an initial response (acquired resistance). Moreover, no targeted treatment options are available for rare forms of melanoma and immunotherapies are less effective in these patient populations compared to patients with cutaneous melanoma (Klemen et al., 2020).

We advocate for melanoma models as critical drivers of discovery, innovation and translation research for next-generation therapies. Melanoma models capture the disease processes in an experimental platform and are necessary for (1) fundamental discoveries in melanoma biology, leading to new therapeutic target and (bio)marker identification, (2) pre-clinical discoveries and insights into therapeutic modalities, (3) understanding and predicting therapy mechanisms of action and response (**Fig. 2**). No single melanoma model can capture the complex landscape of melanoma progression representing all patients, yet a multitude of model systems have together become a cornerstone to the understanding, diagnosis and treatment of melanoma (**Fig. 3**). That animal studies are consequential to melanoma therapy is exemplified by the efficacy of anti-PD-1 and anti-CTLA-4 antibodies to inhibit immune checkpoints in mouse models, leading to auto-immune disease and rejection of transplanted tumors (Iwai et al., 2002; Iwai et al., 2005; Leach et al., 1996; van Elsas et al., 1999; van Elsas et al., 2001). This work transformed the care of patients with melanoma and other cancers, and led to the award of a Nobel Prize for Honjo and Allison (Hodi et al., 2010; Robert et al., 2015).

Here, we discuss state-of-the-art melanoma models, identify five of the most significant challenges for new melanoma therapies and discuss how models are necessary to help overcome them. Further, we identify necessary advances to improve models, now and in the future, to make breakthroughs in these critical areas.

Melanoma Models

Genetically engineered mouse (GEM) models

Mouse models of melanoma genetically engineered to harbor one or more known drivers of subtypes of human melanoma have greatly enhanced our understanding of the functional genetics underlying melanoma formation and progression (**Fig. 2**) (Bardeesy et al., 2001; Bonet et al., 2017; Marsh Durban et al., 2013; Olvedy et al., 2017; Perez-Guijarro et al., 2017; You et al., 2002). GEM melanoma models have more physiological growth rates relative to other models and are useful for evaluating therapies and sunscreens. Accordingly, numerous studies have evaluated small molecule drugs including $BRAF^{V600E}$ and MEK inhibitors with response characteristics resembling human melanoma. The emergence of ICIs as standard melanoma therapy has raised the possibility that GEM melanoma models could be used to evaluate immune-oncology therapies (Galvani et al., 2020; Perez-Guijarro et al., 2020). GEM models capture the intricacies of the tumor immune response and microenvironment for spontaneous melanomas, biological responses that are otherwise difficult to study due to the complexity and variation within human patient cohorts. Genotype-specified GEM models will undoubtably continue to be useful for uncovering mechanistic aspects of melanoma response and resistance to targeted therapy (Dankort et al., 2009; Das Thakur et al., 2013; Deuker et al., 2015; Dhomen et al., 2009; Marsh Durban et al., 2013; Petit et al., 2019; Vredeveld et al., 2012).

Given that melanomas arise from the melanocyte lineage, GEM models designed to study melanocyte stem cells and development have been crucial for providing modeling tools, and for demonstrating the dependence of melanomas on developmental lineages (Conde-Perez and Larue, 2014; Delmas et al., 2007; Dorard

et al., 2017; Nishimura et al., 2005; Shakhova et al., 2012; Sun et al., 2019; Zurkirchen and Sommer, 2017). Emerging gene editing technologies will improve GEM models, since they can replace current models that inactivate genes simultaneously with inducible, temporally controlled changes, recapitulating the accumulation of genetic changes over time commonly seen in human disease. Unlike humans, mice have fur and mouse melanocytes are mainly located in hair follicles; however, this limitation can be overcome. HGF-based GEMs demonstrate “humanized” skin, in which mouse cutaneous melanocytes are ectopically localized at the epidermal/dermal junction as well as hair follicles (Noonan et al., 2001). Alternatively, melanomagenesis can be induced on the tail or ear, which are inhabited by interfollicular, cutaneous melanocytes, and are useful for studying tanning and melanoma development (Cui et al., 2007; Glover et al., 2015; Kohler et al., 2017).

Ultraviolet radiation (UVR) exposure is a major risk factor for human melanoma, and the cause of genome wide mutations (Cancer Genome Atlas, 2015). UVR has been incorporated into GEMs to address tanning and burns, genetic risk factors, and to test sunscreens and tanning chemoprevention (Cui et al., 2007; D'Orazio et al., 2006; Day et al., 2017; Hennessey et al., 2017; Mujahid et al., 2017; Nguyen and Fisher, 2019; Noonan et al., 2001; Viros et al., 2014). Mouse models provided evidence to support the concept that UVR burns in childhood increase risk of melanoma in adulthood (Klug et al., 2010; Noonan et al., 2001). UVR models also demonstrated the molecular mechanisms linking DNA damage to tanning, science which underpins public health advice on indoor tanning (Fisher and James, 2010). Notably, despite the importance of the $BRAF^{V600E}$ mutation in nevi and melanoma initiation, it is not itself a UVR signature mutation. However, $BRAF^{V600E}$ mutant mice were critical to demonstrate that even mild sunburn is sufficient to cause melanocyte clonal expansion, and that repeated mild UVR exposure causes melanoma (Dhomen et al., 2009; Viros et al., 2014). These models recapitulate the UVR-induced genetic signature and recurrent

mutations prevalent in human melanoma (Trucco et al., 2019). Together, GEM UVR models have directly demonstrated the efficacy of sunscreen and provided the molecular evidence to support public health melanoma prevention campaigns.

Syngeneic models

Syngeneic transplantation models have long been a workhorse for pre-clinical testing and offer the advantages of relative ease of use, low cost, and fast turnaround time. However, model-specific features should be considered. The B16 mouse cell line has been widely used for decades to model anti-cancer immune responses in mice with intact immune systems, but typically curative responses are not observed unless a vaccine approach is incorporated. In recent years, several new genetically defined syngeneic transplantation models have been developed from GEM melanoma models, termed GEM-derived allografts (GDAs) (**Fig. 3**). Melanomas used to derive cell lines typically arose on mice backcrossed to C57BL/6 and the lines are typically transplantable into C57BL/6 host mice without rejection (Jenkins et al., 2014; Meeth et al., 2016). With the advent and success of immune therapies in melanoma and other cancers, models with enhanced immunogenicity that exhibit curative responses to ICIs have been developed (Wang et al., 2017; Zelenay et al., 2015). Some of these have involved UVR or other forms of mutagenesis followed by isolation and characterization of single cell-derived clones (Wang et al., 2017; Wolf et al., 2019). Other approaches have utilized expression of model antigens like ovalbumin or LCMV antigens to enhance immunogenicity and enable analysis of antigen-specific T cell responses using reagents that were previously defined for these antigens (Lelliott et al., 2019). A comparison of the immunogenic features of a variety of syngeneic melanoma models was recently completed (Perez-Guijarro et al., 2020).

Patient derived xenografts (PDX) models

PDX models, in which patient cancers are grown in an immunocompromised mouse, have revealed important aspects of melanoma evolution and drug resistance mechanisms. PDX models can accurately predict response to targeted therapies, (Fiebig et al., 1984; Sanmamed et al., 2016), and can be used to explore the role of primate-specific genes, such as lncRNAs in melanoma that are not conserved between species and are enriched for genetic alterations related to cancer (Leucci, 2018; Leucci et al., 2016).

A sufficiently large collection of PDX models can capture much of the clinical, histologic, and genetic diversity of human melanoma compared with GEM models, which recapitulate only a limited number of genetic alterations. Krepler et al. described a collection of 459 PDX models, derived from 384 patients (Krepler et al., 2017). Most of these samples were derived from metastatic lesions, representing all major genetic drivers (e.g., *BRAF, NRAS, NF1)* and subtypes of melanoma (e.g., cutaneous, mucosal, acral, uveal). Moreover, PDXs obtained from patients who had progressed on ICI and targeted therapies were represented, and a subset of these models was subjected to in-depth molecular analyses (Garman et al., 2017), thereby offering an invaluable resource for drug development and translational research. Importantly, parallel sequencing analysis of primary patient tumor samples and patient-derived cell lines showed that the PDXs generally faithfully preserve genetic lesions present in the original tumor (Kemper et al., 2015; Woo et al., 2019). These models were used to establish pre-clinical efficacy of multiple interventions in the context of targeted therapy-resistant melanoma, such as PI3K pathway, MDM2, and BET inhibitors (Echevarria-Vargas et al., 2018; Krepler et al., 2017; Krepler et al., 2016).

PDX models also have limitations. Following the initial *in vivo* passage, the tumor stromal component becomes that of the mouse host, rather than the original human patient, potentially complicating studies of tumor-stroma interactions. Also lost are species-specific signaling interactions between mouse and human ligands and

receptors. Moreover, mouse genomes encode retroviruses capable of infecting human cells, potentially serving as a driver of insertional mutagenesis in the engrafted tumor cells (Naseer et al., 2015). Conversely, the immunocompromised mice used as PDX hosts are highly susceptible to infection by human pathogens that may be present in human tumor samples (Manuel et al., 2017). PDX models of uveal melanoma, notoriously resistant to targeted and ICI therapies, have proven to be challenging, highlighting the need for further technical advancements in PDX approaches for this melanoma subtype (Chua and Aplin, 2018). Notably, current PDX models are limited to analysis in immunocompromised host mice, preventing the testing of immune-based therapies. Many of these issues could be overcome when more robust and tractable humanized mouse models are developed, which is currently a daunting challenge (see below).

Organoids as melanoma models

To complement *in vivo* studies in mice, a variety of novel *ex vivo* approaches have been developed. Upon *in vitro* culture in defined conditions, cancer cells derived from primary patient material organize into organoids that morphologically and functionally mimic significant aspects of the original tumor (Tuveson and Clevers, 2019). Organoids can be expanded, passaged and banked for further use if maintained under appropriate conditions, and can be genetically and phenotypically profiled to identify outstanding candidates for *in vitro* drug screening. Unlike other diseases such as colon cancer, limited experience so far exists utilizing melanoma organoids for drug screens and pre-clinical studies (Tuveson and Clevers, 2019; van de Wetering et al., 2015). However, emerging evidence suggests that organoids can be readily generated from melanoma biopsies and might even be supplemented with autologous immune cells for evaluation of immunotherapeutic responses (Votanopoulos et al., 2019). In fact, the National Cancer Institute is building repositories of well annotated organoids from

melanomas and other diseases that will become available to the community (https://pdmr.cancer.gov).

Other advanced in vitro *melanoma models*

Alternatives for advanced non-animal, *in vitro* melanoma modeling and experimental therapeutics include skin reconstructs, tissue explants and 3D-spheroids. Skin reconstructs co-culture various primary and/or established cell types embedded in collagen such as fibroblasts, endothelial cells and keratinocytes in addition to melanoma cells (Li et al., 2011; Satyamoorthy et al., 1999). Advantages include the 3D- and multi-cell type nature of the reconstruct that more closely mimics *in vivo* features. However, skin reconstructs have limited scalability for multi-cellular processes, can be difficult to generate using allogeneic material and lack immune or other key cells of the tumor microenvironment (TME).

Tissue explants are small pieces of surgical specimens resected from patients that can be cultivated for a limited time (Powley et al., 2020). Such explants contain all components of a patient's cancer *in situ* at the time of culture initiation. Different protocols exist for culture, but over time, this composition will likely change significantly. A clear advantage of this system is the native cellular composition, with autologous material and cells of all kinds present on site for analysis and drug testing. In colon cancer, for example, drug candidates that later were found to have activity in patients could be successfully identified using this approach (Halama et al., 2016). Tissue explants has been shown to work for melanoma (Berezhnaya et al., 2006; Hegerfeldt et al., 2002) but with substantial heterogeneity in yield, indicating that additional studies will be required to expand their utility.

Zebrafish melanoma models

Melanocyte development and the genetic basis of melanoma is shared between fish and human (**Fig. 2**) (Mort et al., 2015). Zebrafish have epithelium-associated melanocytes, and in zebrafish models of cutaneous melanoma, tumors develop near the epithelium, as is typical of human disease. Rapid and scalable melanocyte-specific transgenesis can overexpress or knockout genes in tumor-prone strains (Ablain et al., 2018; Ceol et al., 2011; Patton et al., 2005), enabling modeling of human melanoma genetics. More recently, an electroporation method called TEAZ has been developed to introduce transgenes directly into the skin of living zebrafish, making temporally defined manipulations of melanocytes and melanomas possible (Callahan et al., 2018).

The small size of zebrafish embryos enables phenotype-based screening of drug libraries and drug-led therapeutic development (MacRae and Peterson, 2015). Drug administration in zebrafish is simple and inexpensive because animals can be soaked in low volumes of drug-laden solutions, making screens and combinatorial tests of drugs straightforward. Notably, zebrafish studies have highlighted transcriptional elongation as a novel therapeutic target in melanoma and led to clinical trials (Johansson et al., 2020a; Santoriello et al., 2020; Tan et al., 2016; White et al., 2011). Drug discovery approaches can now encompass patient-derived tumors engrafted in zebrafish (Yan et al., 2019).

One of the strengths of the zebrafish system is that melanomas can be directly imaged using confocal and other high-resolution modalities in real time (Heilmann et al., 2015; Kaufman et al., 2016; Travnickova et al., 2019; White et al., 2011; White et al., 2008). By incorporating cell type-specific labels onto melanoma backgrounds, interactions between melanoma cells and other cells can be monitored, including innate and adaptive immune cells (zebrafish have a full representation of all key components of innate and adaptive immunity), other skin-resident cell types and stromal cells (Feng et al., 2012; Roh-Johnson et al., 2017). The ability to edit genomic loci with precision

and in a temporal-specific fashion remains challenging, although recently precise genome editing has been performed in zebrafish (Prykhozhij and Berman, 2018). Experiments in zebrafish have pioneered the development of lineage tracing technologies such as zebrabow and gestalt (McKenna et al., 2016; Pan et al., 2013) which, when combined with single-cell analyses, constitutes a powerful approach to the study of tumor clonality and heterogeneity.

Melanoma Challenges

Challenge 1: Metastasis

Metastasis is the spread of tumor cells from a primary lesion to a distant site within the body and is responsible for most cancer-related deaths (Nguyen et al., 2009). In addition to the intrinsic properties of the cancer cell that enable this process, extrinsic cues from the TME of the metastatic site also provide support for the growth of disseminated tumor cells (Fares et al., 2020). To identify new drug targets for melanoma metastases, it is critical we understand intrinsic and extrinsic factors that (1) drive metastasis, (2) dictate tumor cell affinity for specific distant sites, and (3) enable tumor cells to remain dormant at distant sites for long periods of time and resist immune and therapeutic destruction. Extensive analyses of patient samples have been invaluable for addressing these questions by providing a plethora of data and hypotheses for further validation and mechanistic study in experimental model systems (Cancer Genome Atlas, 2015; Lauss et al., 2016).

Time to metastatic progression. GEM models can mimic human melanoma metastatic progression and have provided great insights into factors that promote metastasis to distant sites such as the brain, including CCR4 (Klein et al., 2017) and AKT (Cho et al., 2015). A key issue with current animal models is that in order to be

tractable, primary melanomas require relatively short latency, which does not provide time to develop metastases. Developing models with longer latency for melanoma development is one option but makes the practicality of the experiments challenging. Resection of a rapidly growing primary melanoma will buy some time for metastatic progression, but this approach is not always sufficient (Day et al., 2015). A potentially faster alternative would be to create mouse models that permit manipulation of gene expression in somatic cells, such as those based on the viral RCAS/TVA system with TVA expression controlled by a melanocyte-specific promoter (Kircher et al., 2019). The RCAS/TVA system enables more rapid, high-throughput analyses of specific genes than can be achieved with GEM models developed by germline mutagenesis. Evolving CRISPR/Cas9 technology will also make it possible to genetically manipulate somatic cells in juvenile and adult inbred mice. Although tracking tumor heterogeneity and rare cell subpopulations is challenging, the novel methodology CaTCH (CRISPRa tracing of clones in heterogeneous cell populations) provides new opportunities to study clonal evolution in cancers (Umkehrer et al., 2020).

Metastatic initiation and dormancy. Modeling metastasis is a major challenge because in patients it can occur rapidly and be detected early, or it can take decades following long periods of dormancy (Aguirre-Ghiso, 2018). Recent long-term studies that show that while Stage I melanoma patients have good outcomes within the first 5 years, 15-29% of patients with Stage I melanomas die from metastasis up to 20 years following initial treatment (Lo et al., 2018). Unlike humans, animal models have short lifespans and models need to be re-designed to capture the biology of dormancy *in vivo*. Incorporation of creative intravital imaging approaches would enhance our ability to employ animals as models for dormancy.

Capturing the live dynamics of disease spread or response to therapy requires detailed spatiotemporal observation of the interactions between cancer cells and their microenvironment, an approach that is particularly powerful when high-resolution

intravital imaging of fluorescently labeled melanoma cells is performed in semi-transparent zebrafish (van Rooijeb et al., 2017). Combined with microenvironment cell-specific knockdown, this allowed the identification of keratinocyte-derived endothelin 3 as a metastasis-promoting factor that regulates phenotype switching of melanoma cells between invasive and proliferative states (Kim et al., 2017). Further, upon invasion into adipocyte-rich environments, lipid uptake is dependent on the melanoma expressed FATP transporter and blocking lipid transfer inhibits tumor growth and dissemination in zebrafish (Zhang et al., 2018).

A novel advance in mouse modeling is the “MET Alert” mouse, engineered with a VEGFR3-luciferase reporter expressed specifically in lymphatic vessels and that reveals metastatic dispersion through lymph vessels (Martinez-Corral et al., 2012; Olmeda et al., 2017). Live imaging of the lymphatic system in these mice reveals the lymphangiogenic pre-conditioning of distant sites to melanoma metastatic niches by the cytokine midkine (Olmeda et al., 2017). This induction at distal pre-metastatic niches is uncoupled from lymphangiogenesis at the primary melanoma, enabling visualization of a whole-body, systemic response to metastatic lesions. Capturing the live dynamics of metastatic “seeds” and the homing to pre-conditioned metastatic niches are critical steps for understanding how metastatic cells survive and may help identify biomarkers to assist in disease management in patients.

Melanoma organotropism. Melanomas metastasize to specific organ sites, including the skin, lung, liver, brain, bone and intestines. PDXs can model the specific metastatic spread observed in an individual patient (Quintana et al., 2008), and metastases from GEM melanomas can also retain the ability to target specific sites upon transplantation. PDX models harbor the inter- and intra-tumor heterogeneity that is frequently found in patient tumors, which likely plays a role in site-specific metastasis. And while GEM-derived melanoma cells have the same engineered genetic aberrations, heterogeneity can be incorporated by exposing mice to relevant, mutagenic dose of UVR (Noonan

et al., 2001; Viros et al., 2014). Current PDX models require immunocompromised mouse hosts, limiting their usefulness in analyzing extrinsic metastatic factors which could be critical for organotropism. In contrast, GEM models have a fully functional immune system, and appropriate tumor-host interactions.

Tumors derived from GEM and other models of human melanoma can also be “trained” to home to selected organs through a series of transplantations. Josh Fidler discovered that by resecting a metastatic lesion from a specific organ, he could enhance the frequency of metastasis to that organ in a second round of subcutaneous transplantation (Fidler, 1973). After several cycles of this transplantation strategy, melanomas could be trained to target specific organs with high efficiency. Specific unmet needs in melanoma metastasis include the creation of additional models for melanoma dissemination to the brain, which tends to be more resistant to currently approved therapies and a site where patients often relapse, and models that accurately mimic tumor cell dormancy at metastatic sites. Overcoming the challenges of understanding and eventually treating or preventing metastatic melanoma will benefit from novel melanoma models that permit live imaging of specific steps in the metastatic process at the whole-body level (Olmeda et al., 2017) and at single cell resolution (Benjamin and Hynes, 2017).

Challenge 2: Drug resistance

Most patients with BRAF mutations often initially show response to BRAF inhibitors, yet the melanoma often recurs only a few months after starting treatment. In 58% of cases the putative resistance mechanism is linked to MAPK pathway mutations that overwhelm the BRAF inhibitor therapy (Johnson et al., 2015). Drug resistance is also seen in patients receiving ICI, an important point that deserves attention in future

studies. It is therefore essential to gain a deeper understanding of the mechanisms of intrinsic and acquired resistance to current therapies.

Primary resistance. Most GEM melanoma models include dominant BRAF or RAS mutations, and respond to MAPK-pathway inhibition (Dankort et al., 2009; Perez-Guijarro et al., 2017; Xiao et al., 2019). However, it is now clear that many infrequent spontaneous genetic alterations confer conditional adaptive growth or survival advantages. Further, in 39% of cases resistance was not accounted for by any validated mutations but displayed transcriptomic alterations (Cancer Genome Atlas, 2015). Thus, research may be well served by focusing on identifying the pathways, such as changes in autophagy, mitochondrial metabolism, or epigenetic modifications, that drive resistance, rather than on studying single mutated genes. For example, therapy that confers sensitivity to MAPK-pathway inhibition has recently shown efficacy in conjunction with the autophagy inhibitor, chloroquine, in pancreatic ductal adenocarcinoma and in other cancers including NRAS-driven and uveal melanoma (Kinsey et al., 2019; Truong et al., 2020). Further, targeting the NAMPT→NAD+ metabolic pathway restores sensitivity of resistant cells to BRAF inhibitors (Audrito et al., 2018; Ballotti et al., 2019; Ohanna et al., 2018).

Addressing innate resistance to current targeted therapies requires models that are driven by non-BRAF/RAS mutations (Perez-Guijarro et al., 2020; Scahill et al., 2017; Travnickova et al., 2019). Moreover, models are needed that reflect specific transcriptional subtypes that are not linked to driver mutations and are predictive for patient outcomes (Kawakami and Fisher, 2016). For example, zebrafish models of the MITF-low transcriptional signature, which is associated with early resistance to targeted therapy, are independent of BRAF mutations (Muller et al., 2014; Travnickova et al., 2019).

Adaptive resistance. Resistance can arise from pre-existing rare tumor cells that are selected by the therapy, or from resistant cells as an adaptive response to therapy either through newly occurring mutations and/or the activation of adaptive responses (Boumahdi and de Sauvage, 2020). Unbiased genome wide screens in cells have been instrumental in the identification of acquired drug resistance mechanisms to targeted and immune checkpoint therapies (Kong et al., 2017a; Vredevoogd et al., 2019). Exemplifying the importance of metabolism in drug resistance mechanisms, Bernards and colleagues demonstrated MAPK-pathway resistance is associated with drug-induced increased levels of ROS, making resistant cells selectively vulnerable to further increases in ROS (Wang et al., 2018).

Adaptive resistance mechanisms are also problematic for immunotherapy, with >60% of melanoma patients progressing on combination immunotherapy. Transcriptional signatures that characterize immunotherapy resistance and predict patient outcomes have been identified in human samples and using syngeneic mouse models (Hugo et al., 2016; Peng et al., 2016; Perez-Guijarro et al., 2020). Modeling resistance mechanisms *in vivo* is highlighted by the rationale for combining targeted therapy with immune based therapies. For instance, upon MAPK inhibition macrophages secrete TNFα to promote melanoma growth; inhibiting TNFα signaling rescues this effect by targeting not only the melanoma cells, but also the microenvironment (Smith et al., 2014).

Models are also required to capture the dynamic cellular heterogeneity that contributes to therapy resistance (Arozarena and Wellbrock, 2019). This is directed in part by MITF, a critical regulator of melanoma cell-intrinsic phenotypic plasticity that facilitates adaptive cellular reprogramming and intra-tumor heterogeneity (Goding and Arnheiter, 2019; Rambow et al., 2019). In particular, a melanoma subpopulation, featured by low MITF/high AXL is considered a drug-resistance phenotype (Muller et al., 2014; Ohanna et al., 2013); AXL-targeted antibody drug conjugates are currently in clinical trials for

different types of solid tumors including melanoma (Boshuizen et al., 2018). The concept of melanoma cell phenotype switching has been proposed to push invasive melanoma cells towards a differentiated cell state where they are sensitive to a tyrosinase dependent pro-drug (Saez-Ayala et al., 2013). Other cell states are distinguished by differences in cell cycle and stem-cell characteristics, including a slow-cycling JARID1B high sub-population that is enriched upon drug treatment and can be targeted by inhibition of mitochondrial respiration (Roesch et al., 2013).

PDX models may be superior for modelling resistance to targeted agents (Krepler *et al*., 2017), in that they faithfully recapitulate acquired resistance and melanoma patient responses to targeted therapy (Rambow *et al*., 2018) and have also been used to show increased dependency of drug-tolerant lesions on mitochondrial translational machinery (Vendramin et al., 2020). Crucially, PDXs generated from therapy-refractory patients can be leveraged to elucidate mechanisms of resistance and define alternative or second-line treatments. Using PDX models, ER translocation of the MAPK pathway was shown to lead to ERK reactivation and protective autophagy, new druggable targets for therapy resistance (Ojha et al., 2019).

***Targeting residual disease*.** As part of modelling adaptive resistance, there is a need to model the cell types and states that emerge at the residual disease site, including the “persister” cells and a pre-conditioned tumor site (Marine et al., 2020). Persister cells represent a small sub-population of cancer cells that may not necessarily have acquired genetic mutations that drive resistance, and their plasticity can be characterised by metabolic, transcriptional and translational states. For example, following exposure to BRAF and MEK inhibitors, melanoma persister cells reversibly remodel their translational program to survive in a quiescent state, and targeting the eIF4A RNA helicase counteracts this reprogramming to the detriment of persister cell survival (Shen et al., 2019). Single-cell RNA-sequencing of *BRAF*-mutant PDXs was used to identify four BRAF/MEK inhibitor-tolerant residual subpopulations, with one

subpopulation contributing to disease recurrence featuring MITF low-to-no activity and regulated by RXR-gamma (Rambow et al., 2018). RXR-gamma antagonist in combination with BRAF/MEK inhibitor treatments was shown to both improve bulk tumor regression and target the adaptive resistant state to prevent recurrence. Understanding the complex milieu of the heterogeneous states of persister cells with their microenvironment surroundings will be bolstered by models enabling live imaging coupled with single cell technologies of the residual disease state, such as in zebrafish (Travnickova and Patton, 2020; Travnickova et al., 2019). The concept of targeting the bulk of the tumor in addition to persister cell states, including adaptive states and those that pre-exist in the primary tumor, will be crucial to delay or even eliminate recurrent disease.

Challenge 3: The immune response

Most patients do not respond to immune-based therapy and/or are affected by adverse toxicities. Immunocompetent animal models allow for in depth analyses of pending questions about the immune microenvironment of both primary and metastatic melanomas, as well as local and systemic responses at central and peripheral sites. Models should be tailored to specific questions and their physiological relevance defined. Key unmet needs include improved understanding of how melanomas evade immune recognition during tumorigenesis, especially at anatomical sites such as the eye, acral surfaces (palm and sole) and the mucosa. Aspects of metastatic tropism to different organs, particularly the brain, is unclear. Models need to address the complex role of the immune system as conditioned by exosomes, immune responses in the tissue, and the role of local immunity and systemic inflammation on the pre-metastatic niche. Further, immune-related adverse events (irAEs) that now affect up to 30-40% of patients have yet to be addressed in model systems.

Immunogenicity. Reflecting patient responses, $BRAF^{V600E}$ GEM models mount a mixed response to anti-PD-1 checkpoint blockade, some with significant responses (Galvani et al., 2020). However, as in patients, many mice fail to respond. It is thought that low mutation rate and associated neoepitope burden in most GEM melanoma models account for the observed lack of response, and indeed $BRAF^{V600E}$/UVR models with higher mutation burdens are associated with better immunotherapy responses. However, increased mutations alone cannot fully explain ICI responses, as $BRAF^{V600E}$/UVR melanomas are associated with stromal remodeling and reduced tumor cell proliferation, a response shown in patients who benefit from sustained immunotherapy response (Galvani et al., 2020).

Progress has also been reported in harnessing the predictive value of GEM models to ICIs across human cutaneous melanoma subtypes (Perez-Guijarro et al., 2020). The use of a pre-clinical platform consisting of GEM models representing major human cutaneous melanoma genetic subtypes, in conjunction with patient datasets and computation data analytics, has uncovered key determinants of ICI efficacy and a melanocytic plasticity signature (MPS) able to predict ICI responses (Perez-Guijarro et al., 2020). This approach demonstrates the potential of GEM models, especially when used in conjunction with sophisticated computational methodology, and their ability to elucidate mechanisms associated with drug response and resistance, and to inform clinical melanoma trials (Perez-Guijarro et al., 2020).

New models are being developed to understand why many patients are unresponsive to ICI, often suffering side-effects associated with an autoimmune disease, while others benefit from sustained long-term cancer suppression (Chamoto et al., 2017; Kumar et al., 2020). New mouse models should avoid artificial antigens, such as ovalbumin and viral antigens, and instead stimulate immune response using more relevant endogenous neoepitopes (e.g., through UVR) (Galvani et al., 2020; Perez-Guijarro et al., 2020; Wang et al., 2017), or through models that create inducible

neoantigens *de novo* (Damo et al., 2020). Such models more accurately reflect the antigenic profile of human melanoma, enabling the generation of reagents for characterizing epitope-specific responses.

Humanizing immune systems in models. PDX models have limited utility for pre-clinical testing of ICIs or evaluating irAEs because human tumors must be grown in immune-compromised mice to prevent rejection. The most popular host is the NSG mouse strain (NOD/SCID/IL-2rγ^{null}), with defects in both adaptive and innate immunity, including an absence of natural killer (NK) cells (Ito et al., 2002). Additionally, the mouse immune system is substantively different from its human counterpart, making direct translational interpretation challenging. Mice with humanized immune systems can help to overcome these limitations. Two mouse strains, MITRG and MISTRG, have been engineered to express several human cytokines from the corresponding endogenous mouse loci (Rongvaux et al., 2014). Once engrafted with human CD34+ hematopoietic stem cells, these models are permissive for development of NK cells, macrophages, and monocytes, which have proven challenging to reconstitute in other systems (Rongvaux et al., 2014). Using this approach, a human melanoma xenograft was found to be infiltrated by immunosuppressive myeloid cells, promoting tumor growth. However, this model does not reconstitute a humanized adaptive immune system. Developing a mouse model with functional mature human B- and T-lymphocytes represents a major technical challenge but remains a high priority. Very recently, humanized mice challenged with HLA-matched melanoma cells demonstrated a critical role for mast cells in mediating resistance to anti-PD-1 antibody therapy (Somasundaram R, in press). Because T-lymphocytes require thymic education, generation of a fully humanized immune system in mice will likely require implantation of thymic tissue using stem cells from the melanoma patient that produced the PDX.

Modeling biological heterogeneity. Understanding the plasticity of melanoma cells and its relationship with microenvironment heterogeneity is critical to understanding immunotherapy resistance. These include, but are not limited to, the (lymp)angiogenic vasculature, stroma (e.g., fibroblasts, adipocytes), tumor-associated myeloid cells, and various immune cells. Other variables that may regulate the host immune response include aging, diet, exercise, sex, and the microbiome. Models are needed that recapitulate known mechanisms of ICI resistance, including loss of MHC I presentation and reception of IFN-γ signaling. In addition, unbiased models and approaches, including CRISPR-based screening, should be developed to effectively mimic resistance to immunotherapy (Vredevoogd et al., 2019).

Modeling the heterogeneity commonly seen in patient melanoma cells themselves represents a tremendous challenge. Within any one patient's melanoma exists a myriad of tumor cells with varying mutations, transcription patterns, and phenotypes, each potentially responding differently to immunotherapy. Beyond that, the human population constitutes the most heterogeneous genetic background imaginable. In contrast, animals used as models are typically largely genetically identical, and their melanomas tend to be much more homogeneous, arising from the engineering of dominant oncogenic mutations and harboring fewer spontaneous mutations. The collaborative cross, a large panel of newly developed inbred strains of mice, has been created to add genetic diversity to melanoma models (Ferguson et al., 2015). Moreover, tumor-bearing animals are bred and raised under strictly controlled conditions, deviating dramatically from the multitude of environmental factors to which humans are exposed daily. UVR is being used in modeling to help compensate for this, and genetically defined mice can now be imbued with a natural, diversified microbiota, through the creation of so-called "wildling mice" (Rosshart et al., 2019); future GEM models will be adapted to harbor such a natural microbiome as well.

Characterization and standardized reporting. Maximizing the utility of any newly developed model requires adequate characterization and standardized reporting of the immune response, and widely available access to such information. This includes standardization on reporting time of tumor growth, aiming to generate tumor regression rather than tumor growth inhibition, and long-term or sustained responses. This standardization should include Kaplan-Meier plots and survival curves as well as granularity in reporting individual mouse responses using spider and spaghetti plots. Newly developed animal melanoma models should be fully characterized with regard to immune profiles, including cytotoxic CD8 T cells, regulatory T cells, B cells, macrophages, and dendritic cells. These profiles may vary across genetic backgrounds. Additionally, the immunogenicity of the models should be characterized, including the relative antigenicity of the melanomas, the frequency of spontaneous regression, the affinity and avidity of the immune response to the tumor, the repertoire of the T cell receptors that recognize the melanoma cells, and the repertoire of MHC class I and II epitopes presented by the melanoma cells. These steps will elucidate the baselines of immune responses in models and can be directly compared with human disease. Importantly, these analyses may also help us to understand the irAEs associated with current immunotherapies.

Challenge 4: The impact of age and environmental exposures

Age remains the greatest risk factor for cancer development overall (Fane and Weeraratna, 2020). Aging provides time for premalignant cells to accumulate mutations, such as those from UVR in cutaneous melanoma, and epigenetic changes that favor cancerous progression and immune escape. The malignant cells and their TME, must adapt to support tumor survival and growth at primary and metastatic sites. The adaptations arise over time from pressures in the TME and the physiologic

macroenvironment of the organism. Similarly, the TME itself ages, and this may place pressures on initiated cancer cells, driving them to enhanced malignancy (Fane and Weeraratna, 2020).

A notable feature of the aged TME is the senescence associated secretory phenotype (Faget et al., 2019). Senescence, the loss over time of a cell's ability to divide, and the cornucopia of molecules secreted by a senescent cell, usually a fibroblast, has been used as a measure of aging. Currently, many models of aging and cancer are based on acceleration of senescence and have led to important insights into the role of senescence and cancer (Demaria et al., 2014; Luo et al., 2016). Overall, such models allow for the examination of pressures placed upon a developing, dormant, or progressing cancer as the TME and organism age (Fane and Weeraratna, 2020).

Natural aging and age-appropriate models. Addressing questions related to the dynamic adaptation of both the cancer cells and TME over time in response to natural aging or environmental factors requires inducible, site-specific models that capture the long latency of human melanoma and can be examined at different ages. Evidence points to older patients with melanoma responding better to ICIs compared to younger patients (Kugel et al., 2018), but *in vivo* studies of melanoma tend to use 6-8 week-old mice, which corresponds to ~20 year old humans, and developing/young-adult zebrafish. However, subcutaneous implantation of melanoma tumors into 12-18 month-old mice can increase melanoma metastasis and affect response to therapy. For example, lipid uptake from aged but not young fibroblasts by FATP2 can drive resistance to BRAF/MEK inhibitors in aged mouse melanoma models, and inhibiting FATP2 overcame resistance in aged mice (Alicea et al., 2020).

Particularly important are age-appropriate models that enable (1) quantitative dissection of adaptive relationships between nascent or dormant tumors and their aging hosts, (2) direct measurement of the progression-promoting crosstalk between

tumor and host and between cancer cells and the TME, and (3) analysis of the response to therapy and age-associated toxicities. One such GEM model for melanoma is MT-RET, whereby mice express the human *RET* transgene in melanocytes, and develop skin melanosis, benign melanocytic tumors and metastatic melanoma in a stepwise manner (Kato et al., 2000). Tumor cells disseminate as early as 6 weeks to the lung; however, they remain slow cycling, and do not develop overt metastases until about 18 months of age. Depletion of CD8+ cells leads to rapid development of metastases (Lengagne et al., 2008), suggesting that the immune microenvironment plays a role in containing the metastatic process. Implanting melanoma cells into syngeneic immunocompetent mice of different ages can also provide important information about the TME during aging. Evaluating these issues within an appropriate age context, given the known epidemiology of melanoma, is critical to accurately model human disease, understand underlying drivers, and identify unique age-related tumor vulnerabilities that may be targeted for patient benefit.

Environmental exposures. Age-associated changes also reflect effects of chronic environmental exposures, such as UVR, diet, smoking, infection, and pollution. Existing models generally fail to consistently incorporate such chronic environmental exposures, which exert direct and indirect effects on tumor growth. Other than UVR (Galvani et al., 2020; Noonan et al., 2001; Shain et al., 2018; Trucco et al., 2019), contributions of environmental exposures to melanoma are not well characterized. A significant challenge in the near future is assessing and accounting for the influence of the microbiota in the gut and other organs in melanoma development and phenotype. Notably, mouse melanoma models were used to demonstrate a causal link between microbiome and response to ICIs, which has since been extended to melanoma patients (Gopalakrishnan et al., 2018; Matson et al., 2018; Routy et al., 2018; Sivan et al., 2015; Vetizou et al., 2015). A clear focus is now on the mechanisms by which the microbiome influences melanoma behavior, which should provide clues

to its influence in clinical care (Li et al., 2019). Ultimately, the superimposition of aging together with chronic environmental exposures in melanoma models will be required to dissect the full complexity of the human disease (Lotz et al., 2020).

Experienced immune systems. In addition to directly contributing to the mutational burden in cancer cells, environmental stressors can also affect the microenvironment and systemic immune response, which can in turn influence melanoma development and progression. Chronic infections acquired during the lifetime, especially common viruses such as human cytomegalovirus (hCMV), Epstein Barr virus, influenza viruses (Kugel et al., 2018), and possibly even COVID-19, shape the immune repertoire and thus can impact immune system-targeted responses to developing or re-emerging tumors and contribute to therapy-induced toxicities. Pathogen-free animals lack this immunological experience and exhibit profoundly altered responses to challenge, such as with a pathogen or the presence of tumor cells, indicating the need for melanoma models with more experienced immune systems (Masopust et al., 2017).

Challenge 5. Rare forms of melanoma

In countries with populations of predominantly European descent, most melanoma diagnoses are of the UVR-induced cutaneous subtypes. However, most cases in many countries in Latin America, Africa, and Asia are acral (occurring on the palms, soles, and in the nail bed), and mucosal melanomas (occurring within sinuses, nasal passages, mouth, vagina, and anus) (Ossio et al., 2017). Less common forms of melanoma also include those that occur in the uvea and conjunctiva of the eye, a subset of pediatric melanomas, and a form that invades the nervous system called leptomeningeal melanocytosis. Due to their low incidence, these non-cutaneous forms of melanoma are often referred to as rare forms of melanoma. Critically, there are no approved therapies specific for rare forms of melanoma.

Rare melanoma genetic drivers. Unlike cutaneous forms of melanoma, and more recently uveal melanomas of the iris (Johansson et al., 2020b), most data indicate that UVR plays a minor role, if any, in the etiology of acral, mucosal, and uveal melanoma (Hayward et al., 2017; Kong et al., 2017b; Liang et al., 2017; Robertson et al., 2017; Yeh et al., 2019). In contrast to cutaneous melanoma, acral and mucosal melanomas are characterized by chromosomal alterations that result in large-scale genomic amplifications and deletions (Ablain et al., 2018; Yeh et al., 2019). The exception is uveal melanoma, most of which have activating mutations in either *GNAQ* or *GNA11*, encoding alpha subunits of G proteins activated by G protein-coupled receptors (Perez et al., 2018; Van Raamsdonk et al., 2009; Van Raamsdonk et al., 2010), and in ~40% of tumors *BAP1*, encoding a deubiquitinase (Harbour et al., 2010).

Mutations in genes characterizing The Cancer Genome Atlas (TCGA) cutaneous melanoma subtypes, namely *BRAF*, *RAS* and *NF1*, are less common in mucosal, acral and uveal melanoma than in cutaneous and conjunctival melanoma, although NRAS is fairly common in mucosal and acral melanoma (Wong et al., 2019). Hence, model generation approaches need to be amenable to rapidly testing potential driver genes both individually and in combinations. One possible approach is the use of embryonic stem cells harboring a melanocyte-specific Cre recombinase allele, Tyr-CreERt2 (Bok et al., 2020). Similarly, transposon-mediated forward genetic screens have been performed to identify drivers of melanoma development, for example genes whose mutation co-operates with $BRAF^{V600E}$, as well as mediators of resistance to BRAF inhibitor therapy and new regulators of metastasis (Mann et al., 2015; Perna et al., 2015). *In vitro* modification followed by the generation of experimental chimeras enables the rapid assessment of driver genes alone and in combination. A parallel approach through genomic alteration analysis of human mucosal melanoma and tissue-specific CRISPR implementation in zebrafish identified SPRED1 as a tumor suppressor in mucosal melanoma in the context of *KIT* mutation (Ablain et al., 2018).

Further increased use of CRISPR technology to knock out multiple genes should enhance progress, and genetic approaches may be supplemented with localized application (e.g., viruses in mice) to induce melanoma at different sites such as the mucosa or uveal tract. Unbiased whole genome CRISPR-Cas9 screens are being used to identify rare melanoma dependencies (Hayward, *personal communication*).

Disease progression. *In vivo* and cell culture-based strategies are useful for rapidly modeling rare forms of melanoma and enable high-throughput drug screening. A few transplantation models of rare melanomas from patients are available (Krepler *et al*., 2017), and the value of these models is demonstrated by their use in pre-clinical testing of palbociclib, an FDA-approved CDK4/6 inhibitor for mucosal melanoma (Zhou *et al*., 2019). Within each rare melanoma disease, we need to capture cell lines that represent the distinct stages of disease progression; for instance, although some uveal melanoma cell lines are available, few are from metastatic tumors. Increased numbers of cell line models representing both early and advanced lesions are still required.

For optimal representation of rare melanomas, cell-based models should incorporate the appropriate aspects of the TME through transplantation into the correct orthotopic site (Stei et al., 2016). This is especially important for the ~50% of uveal melanomas that eventually metastasize to the liver, a more selective metastatic site tropism than observed in other melanoma subtypes (Ozaki et al., 2016; Richards et al., 2020). An imperative is the cataloging and characterization of available rare melanoma cell lines representing the repertoire of genomic alterations. Models of rare forms of melanoma should be optimized to enable *in vivo* imaging for quantitative and spatiotemporal assessment of tumor tropism and growth

Genetic and ethnic diversity. Patient-derived cell lines of rare forms of melanoma exist and are growing in number but have been difficult to derive. For all melanoma research, but especially for rare melanomas, cell-based models need to include

representation from different ethnicities, sites on the body, and genomic profiles to capture the extensive diversity of human melanomas (Ossio et al., 2017). Since rare forms of melanoma are proportionally more common in individuals of Asian, Latin American and African descent, it is important to foster international collaborations to understand the germline contribution to disease presentation and predisposition. Further, the poorer prognosis of patients with rare forms of melanoma is a clear example of health disparity (Klemen et al., 2020). These collaborations will also help improve outcomes through knowledge exchange between researchers and clinicians at major global centers, including those in low- and middle-income countries, who have considerable experience and expertise in these forms of the disease, and where high volumes of patients are seen. At present a resource of acral melanoma PDX and cell lines is currently being developed by researchers in Mexico and Brazil with assistance from a UK-based team.

Veterinary oncology. Pet dogs develop several forms of melanoma, including cutaneous, mucosal, acral, and uveal. Of these canine models, oral mucosal melanoma shows similar histology and has some of the genomic features common to oral mucosal melanoma in humans (Prouteau and Andre, 2019; Wong et al., 2019). In fact, trials in dogs with oral melanoma led to the first federal approval of a therapeutic cancer vaccine for commercial use (Bergman et al., 2003; Grosenbaugh et al., 2011). Pigs and horses may be other animals that could serve as models of rare forms of melanoma (van der Weyden et al., 2020).

Melanoma models: Rising to the Challenge

Across models and challenges, common themes emerge as key opportunities to advance melanoma therapy discovery.

Integrating multiple models: There is tremendous value in conducting a cross-species approach for the development of novel melanoma therapies. For example, the ability to perform high-throughput screens to identify new targets or pathways in resistant cells using CRISPR-Cas9 technology can be performed with organoids or patient-derived cell lines co-cultured with immune, endothelial, and/or fibroblast cells. By screening libraries of potential therapeutics in the CRISPR-modified cells, drugs or drug combinations targeting those vulnerabilities can be rapidly tested. Candidate therapies can then be tested *in vivo* in pre-clinical fish and mouse models, and the impact on melanoma biology studied in the context of a whole animal system. Predictive pre-clinical modeling will also greatly benefit from the integration of multiple models. The advantages of an intensive cross-species (e.g., zebrafish, mouse, human) approach can also be applied to the examination of the TME over time, in various environmental conditions and in response to therapy. Tumor initiation can be evaluated for common mechanisms, such as the induction of the neural crest state, to determine if age and environmental exposures influence melanogenesis pathways. A simplified melanoma genome in models compared to human melanoma can further be exploited to search for new melanoma genes (Ablain et al., 2018; Olvedy et al., 2017; Venkatesan et al., 2018; Yen et al., 2013).

Computational approaches*:* The tools with which we capture large “omics” data are becoming ever more sophisticated, allowing capture of transcriptomic, exome, proteomic, CHIP-sequencing, and ATAC-sequencing data, opening up avenues to analyze potentially rare cell types, subpopulations, or limited patient tissue. Innovative analyses will enable direct comparisons between models and human disease. Cross-referencing with normal datasets, including melanocytic or neural crest lineage and the immune compartment, can be used to parse out meaning and patterns (Cao et al., 2019; Marie et al., 2020; Rambow et al., 2015; Rambow et al., 2018; Soldatov et al., 2019; Varum et al., 2019; Zilionis et al., 2019). Evolutionarily conserved

signatures/pathways identified in models have proven useful for establishing which melanoma subtype or process the model represents and for translation to the clinic (Galvani et al., 2020; Johansson et al., 2020a; Kaufman et al., 2016; Marie et al., 2020; Perez-Guijarro et al., 2020; Travnickova et al., 2019; Trucco et al., 2019).

A unified, bespoke approach between biologists and data scientists as to how and what data are generated and analyzed will help answer novel questions. For example, single-cell RNA-sequencing (scRNA-seq) atlases of patient cohorts or melanoma models, generated by collating disparate experiments representing multiple biological contexts, are beneficial for comprehensive analyses. Heterogenous multi-sample data can then be aligned and analyzed using applications such as Conos and cellAlign (Alpert et al., 2018; Barkas et al., 2019).

Melanoma patient datasets should be used to explore translational benefit with patient characteristics, such as patient treatment, mutational subtype, sex, pathological stage, and ethnic origin, taken into account (Lotz et al., 2020). TCGA was revolutionary for its amount of available melanoma patient transcriptomic data (Cancer Genome Atlas, 2015), and now computational approaches that infer the TME component based on bulk sequencing are fully exploiting these data (Jimenez-Sanchez et al., 2019). However, some annotation is incomplete and there is often a lag between the date of tumor staging and collection (Cancer Genome Atlas, 2015; Liu et al., 2018; Marie et al., 2020). Other patient datasets are also available that have their own unique set-up and data collection, including patient-matched whole exome and transcriptome sequencing data (Van Allen et al., 2015); pre-treatment samples (Hugo et al., 2016; Van Allen et al., 2015); matched histopathological analysis (Nsengimana et al., 2018).

With the advent of single-cell sequencing technology, we are able to obtain more information from limited samples from patients and models to better understand intra-tumor heterogeneity (de Andrade et al., 2019; Jerby-Arnon et al., 2018; Li et al., 2019;

Rambow et al., 2018; Sade-Feldman et al., 2018; Tirosh et al., 2016; Travnickova et al., 2019). Models can be used to explore sparse cells and samples, such as micro-metastases, dormant cells and residual disease, which would be difficult to obtain in the clinic. It is also possible to collect sufficient cells for analysis from one biopsy, thereby allowing monitoring of tumor progression on-therapy in real time. This allows melanoma modeling to generate dynamic signatures of tumor progression and therapy response, which can be used in the clinic for personalised medicine applications.

***Quantitative dynamic* in vivo *information*:** Addressing questions related to *in vivo* tumor-stromal crosstalk and dynamic adaptation ideally requires inducible, site-specific models. While this remains a challenge, the careful design of models to ask specific questions and incorporate novel technologies to facilitate quantitative analyses will provide a path forward. Intravital imaging is a mainstay approach by which to examine the dynamic TME. Models that permit live imaging of specific steps in melanoma development, as well as melanoma/stromal interactions, immune responses, and metastatic events will address each of the challenges. Ideally, these should enable imaging at the level of the whole body, entire tumor, and at the single cell level (Hirata et al., 2015). Zebrafish are particularly well suited for intravital imaging; however, there are limitations to its application at some tissue sites, and it remains technologically challenging and resource intensive.

New techniques that provide quantitative dynamic information *in vivo* (e.g., signaling reporters, lineage tracing, barcoding, inducible labeling), paired with computational approaches to predict evolutionary trajectories and receptor/ligand interactions across diverse cell types, will increase throughput and capture spatiotemporal regulation of the tumor niche at primary and metastatic sites. These efforts will be supported by continued adoption of multiplexed image analysis and spatial-omics platforms that are capable of quantitatively localizing relationships within intact tissues, and simultaneously capturing tumor intrinsic and extrinsic heterogeneity. Such assays can

then provide a link to human samples for future validation and data integration across modalities, paramount to developing a complex understanding of the dynamic networks that feed tumor growth.

Improved preclinical modeling: Future development of mice bearing a humanized immune system to host PDX transplants has been discussed, as have the advantages of integrating diverse models and of computational analytics in preclinical approaches. However, in order to fully exploit preclinical models to accurately predict patient responses and inform clinical trial decisions, rigorous conduct and reporting, including appropriate design and sample size, blinding and randomization, and statistical analyses, must be strictly employed to ensure reproducibility (Percie du Sert et al., 2020). Truly informative preclinical trials often require extensive resources and development time, items that are not always readily available to individual academic laboratories. We recommend that grants with ample resources be established to specifically support the development of preclinical models for drug development, including drug efficacy, biomarkers and ADME-tox properties. Moreover, dedicated regional centers of preclinical modeling excellence should be instituted to support and educate researchers who wish to faithfully develop a drug-lead from the discovery phase into the drug development pipeline, and provide critical links with clinical centers and the pharmaceutical industry.

Repositories: A key requirement for realizing the full potential of models is ensuring their widespread availability and dissemination to the research community. Several representative PDX models from the collection described by Krepler et al. have been provided to a commercial vendor for distribution (Horizon Inc/Envigo), and there are plans to enhance the availability of the collection still further via the NCI PDXNet (www.pdxnetwork.org). There is a conceptually similar initiative to make genetically characterized melanoma and other tumor PDXs available in Europe (www.europdx.eu). Melanoma models characterized for copy number alteration and

RNA-seq are hosted by Trace (https://www.uzleuven-kuleuven.be/lki/trace/trace-leuven-pdx-platform) and available through the europdx research infrastructure (https://www.europdx.eu/europdxri-ta). All these models are annotated in the PDX finder (http://www.pdxfinder.org/).

A central repository is needed to provide methodological guidelines for the reporting and analysis of cell lines and animal models. Rigorous standardization, quality control, and genetic characterization when generating PDX models should be emphasized. This should include how to use the models for the implantation of cell lines or for the testing of endogenous genetic modifications. Information regarding the validity of each model for studying the responses of primary and metastatic lesions to targeted therapies and ICIs, as well as irAEs will be important. Caveats to each model (e.g., limited metastatic potential) should also be addressed. This goal includes sharing knowledge on how to use these models for immune assessments, including the window of opportunity for treatment, (neo)adjuvant regimes, vaccination or treatment of established metastases. Details regarding early versus late therapeutic escape will also be informative to the research community.

Repositories will also facilitate progress toward effective therapies for rare melanoma subtypes, which would benefit greatly from generating a catalog of cell lines derived from rare melanomas coupled with various animal models that capture the range of rare melanoma subtypes, the genetic lesions, and the diversity of the patient population. These models can significantly advance our understanding of rare melanoma etiology, disease progression and drug treatment. Importantly, we need to understand the unique drug targets in each subtype within the context of the diversity of the human population in order to develop the best therapeutic strategies.

Conclusions

The use of animal models derived from varied species combined with cell-based models will provide a mechanistic understanding of melanoma occurrence and outcome, generating critically needed insights into new targets and biomarkers to guide novel clinical paradigms. Further, it is increasingly apparent that tissue context regulates immune surveillance, activation, localization, and function, and targets within the TME could provide orthogonal strategies for synergistic therapeutic combinations with ICIs, or mitigation of off-target toxicities. Ultimately, the appropriate use of animal models provides a rapid route, relative to observational human studies, to fully understand and elucidate *in vivo* crosstalk, to differentiate the normal from the pathological, and to characterize the dynamic maladapted processes that drive disease. Finally, we propose that conducting cross-species studies of melanoma biology is advantageous and may provide synergic outcomes. Where models mimic or diverge from human disease, this will focus our use of these systems to probe specific mechanisms relevant to the clinic. In this way, we can identify the unique vulnerabilities that can be targeted and the specific challenges that must be overcome in order to eradicate this disease.

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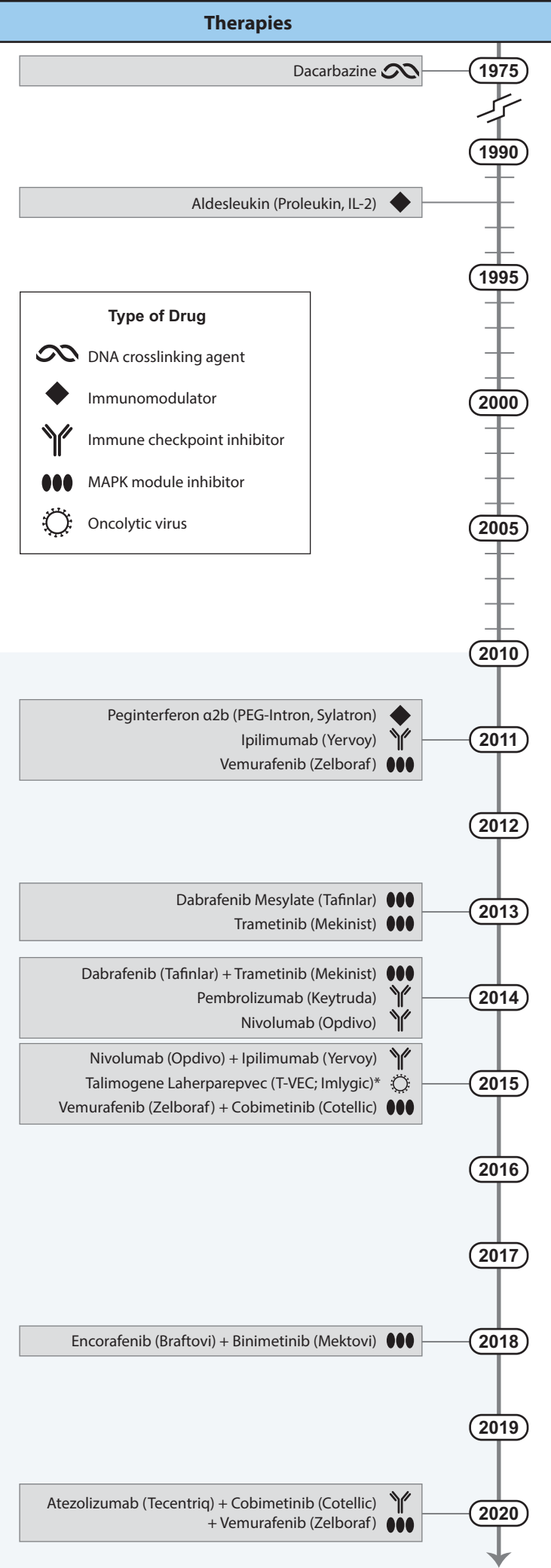
Figure legends

Figure 1: Timeline of treatment approvals by the US Food and Drug Administration for advanced melanoma patients. Thirteen new treatments, including both single agents and combination therapies, have been approved since 2011. 2011 also marked the first approval of an immune checkpoint inhibitor – ipilimumab – which ushered in the modern era of cancer immunotherapy. Several of these new treatments have also been approved in the adjuvant setting (after surgery). Credit: Heather McDonald, Nancy R. Gough, BioSerendipity, LLC.

Figure 2: **Timeline of melanoma models and opportunities for addressing key challenges.** A brief selection of models from mouse, zebrafish, human and other species that have made major contributions to our understanding of melanoma biology, and in the discovery of new therapies. Such models are critical to address the most pressing challenges in melanoma, including metastasis, drug resistance, immune response, ageing and the microenvironment, and rare forms of melanoma. Due to space constrictions, unfortunately only a subset of available melanoma models is presented. Species are indicated by colored circles, and challenges indicated by letters in colored circles. Credit: Heather McDonald, Nancy R. Gough, BioSerendipity, LLC.

Figure 3: **Schematic representation of current melanoma models and the many ways they can be employed to develop new therapies.** Animal models are first generated to resemble their human counterparts as closely as possible, but are also constantly being improved through genetic engineering, environmental intrusions, and/or technical advances. In general, *in vitro* models are more amenable to mechanistic analysis, while *in vivo* models help capture the complex microenvironment and immune responses that contribute to melanoma outcomes. A critical evolving aspect to all animal models is that 'omics' data generated from their study can now be

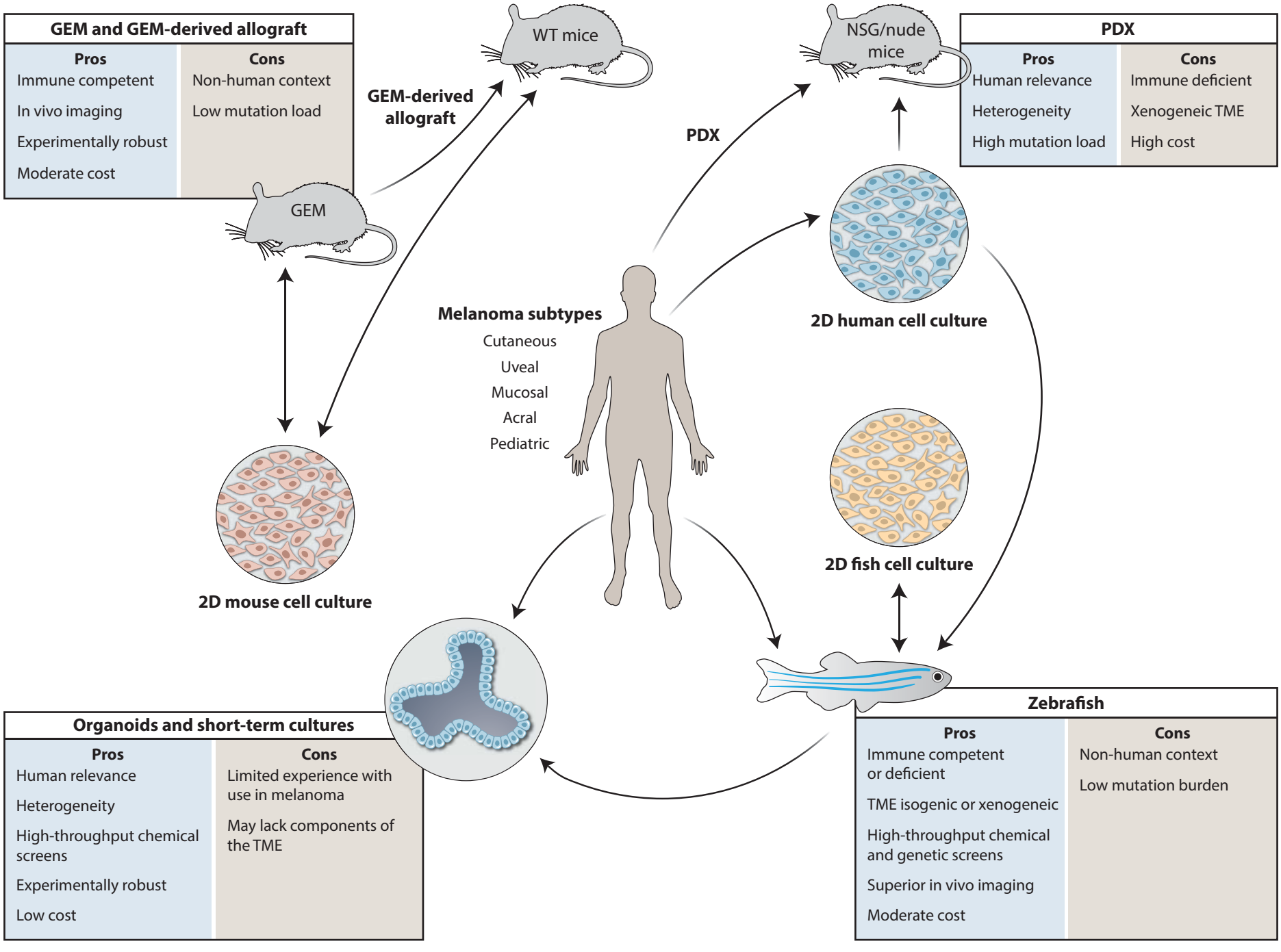
compared directly to comparable human datasets, providing powerful validation of relevance. The establishment of *in vitro* cultures generated from the models and directly from patients (such as organoids) provide additional layers of experimental possibilities. Abbreviations: GEM, genetically engineered mouse; GDA, GEM-derived allograft; HT, high throughput; ME, microenvironment; NSG, NOD scid gamma immunodeficient mouse; PDX, patient derived xenograft; WT, wildtype.


Therapies
Dacarbazine
1975
1990
Aldesleukin (Proleukin, IL-2)
1995
Type of Drug
DNA crosslinking agent
Immunomodulator
Immune checkpoint inhibitor
MAPK module inhibitor
Oncolytic virus
2000
2005
2010
Peginterferon α2b (PEG-Intron, Sylatron)
Ipilimumab (Yervoy)
Vemurafenib (Zelboraf)
2011
2012
Dabrafenib Mesylate (Tafinlar)
Trametinib (Mekinist)
2013
Dabrafenib (Tafinlar) + Trametinib (Mekinist)
Pembrolizumab (Keytruda)
Nivolumab (Opdivo)
2014
Nivolumab (Opdivo) + Ipilimumab (Yervoy)
Talimogene Laherparepvec (T-VEC; Imlygic)*
Vemurafenib (Zelboraf) + Cobimetinib (Cotellic)
2015
2016
2017
Encorafenib (Braftovi) + Binimetinib (Mektovi)
2018
2019
Atezolizumab (Tecentriq) + Cobimetinib (Cotellic)
+ Vemurafenib (Zelboraf)
2020

Organism: Zebrafish · Mouse · Human · Other

Challenge: (M) Metastasis · (D) Drug resistance · (I) Immune response · (A) Age and environment · (R) Rare forms

PMID	Org	Model and Contribution		Challenge	Opportunities and Application to Challenges
6616619		Cell line that establishes reversible nature of melanocyte differentiation	1983	M D A	Foundational cell line for examining phenotypic plasticity in melanoma cells
1846036		First GEM model of cutaneous and ocular melanoma	1991		
1915289		Introduction of *RET*-based GEM melanoma model			
8062242		UV-induced melanoma in transgenic mice → Prevention	1994		
9353252		H-RAS^{G12V} model → Foundational transgenic model of melanoma	1997	M D	Evaluate mechanisms of melanoma genetics and drug response
10721493		3D skin reconstruction models		I A R	Follow melanoma cells in the context of human skin for common and rare (BRAF wildtype) forms of cutaneous melanoma
10440378		First inducible Ras-driven melanoma GEM model showing Ras requirement for tumor maintenance	1999	D I A	Evaluate mechanisms of melanoma maintenance and regression, and interactions with microenvironment
11514604 10430624		B16-BL6 and B16-F10 melanoma cell lines → Efficacy of anti-CTLA-4 immune checkpoint inhibitor therapy		M D I A R	Use in syngeneic mouse models to evaluate metastasis, drug resistance, environmental effects, and immune responses
11565020		Evidence supporting risk of childhood sunburn for developing melanoma → Prevention	2001	I A	Use HGF transgenic mice to evaluate UV effects and immune responses, and to generate melanoma cell lines for syngeneic mouse studies
12384415		Immunodeficient NSG (NOD/SCID/IL2rγ null) mice for melanoma xenografts	2002	M D A R	Laboratory gold standard for immunologically incompatible xenograft studies
15611321 12218188		B16 melanoma cell line → Efficacy of anti-PD-1/PD-L1 immune checkpoint inhibitor therapy		M D I A R	Use in syngeneic mouse models to evaluate metastasis, drug resistance, environmental effects, and immune responses
15694309		BRAF^{V600E} model → Foundational model in zebrafish and first animal model of BRAF-mutant melanoma	2005	M D I A R	High-throughput model system for functional genomics and drug screening
18006687		NRAS^{Q61K} plus stabilized beta-catenin models → Synergy between the Wnt and MAPK pathways	2007	M D A	Evaluate mechanisms of metastasis, test drugs for metastasis prevention, and understand melanoma onset
19282848 19345328		BRAF^{V600E} GEM models → Foundational models in mouse for BRAF-mutant melanoma	2009	M D I A R	Resource for preclinical evaluation and functional genomics
20726949		Evidence for sunscreen protection and shared molecular mechanism in human and mouse → Prevention	2010		
21430780		Neural crest gene expression signature identified → New therapeutic target, clinical trial of leflunomide	2011		
21430779		MiniCoopR model → Second generation model in zebrafish, new therapeutic target (SETDB1)		M D I A R	High-throughput model system for functional genomics and drug screening
22611244		Humanized (myeloid compartment) NSG mice	2012	M D I A R	Second generation, partially immune-competent mouse model for xenograft studies
23123854		red-BRAF^{V600E} mouse model → MC1R deficiency in red-haired individuals increases melanoma risk		D A	Understand how human genetic variation and environmental risk factors contribute to melanoma
24919155		Evidence for role of mild sunburn (UV radiation) in melanoma burden in BRAF^{V600E} GEM and benefit of sunscreen → Prevention	2014	D A	Elucidate UV-induced DNA damage mechanisms and test new therapies
25088201		First use of Collaborative Cross (hundreds of inbred mouse strains from 8 genetically diverse founders) in mouse melanoma modeling		M D I A	Assess role of genetic variation in melanoma development in mouse models using Collaborative Cross
26105199		Comprehensive melanoma PDX collection (Europe)		M D I A R	Gold standard resource for performing fundamental research, preclinical testing, and developing personalized medicine
26282170		Live imaging of metastasis	2015	M D A	Visualize metastatic processes, disease recurrence after treatment, and escape from tumor dormancy
26565903		GEM model of metastasis → Secondary site tropism		M	Evaluate mechanisms of metastasis, test drugs for metastasis prevention, and understand secondary site tropism
26541610 26541606		Mouse melanoma models → Causal link between gut microbiota and response to checkpoint blockade, leading to clinical trials		D I A	Evaluate relationship between therapeutic response, immune responses, and gut microbiota
26823433		Live imaging of melanoma initiation → Major advance in live animal imaging	2016	M D A	Evaluate mechanisms of melanoma initiation and treatment response
27042933		Aged mice → Increased melanoma metastasis		M D I A	Evaluate how aging alters responsiveness to treatment and affects microenvironment to become permissive to melanoma
29570931 27166257		Uveal melanoma model → YAP, not MAPK signaling, as a driver		R	Understand mechanisms of rare form of melanoma and screen potential therapies
29141224 29141225		Comprehensive melanoma PDX collection (US)	2017	M D I A R	Gold standard resource for performing fundamental research, preclinical testing, and developing personalized medicine
28379630		Immunogenic and checkpoint inhibitor-sensitive cell lines (YUMMER1.7 lines)		D I	Resource for evaluating immune-melanoma interactions and testing immune-targeted therapies
28658220		In vivo imaging of lymphovascular pre-metastatic niche → New targets for therapy (midkine, VEGFR) and clinical trial of BO-112		M D I	Visualize microenvironment of pre-metastatic niches during melanoma progression
28614705		Chemoprevention in high-risk model → New target (SIK) and drug lead for prevention		D I A	Assess effectiveness of increasing pigmentation as a prevention strategy
28376192		Mouse model of heritable MITF^{E318K} mutation that predisposes individuals to melanoma		M D A	Investigate the mechanisms of how human germline mutations contribute to nevi and melanoma formation
30385465		Mucosal melanoma model and human tumor genomics → First mucosal melanoma model, new therapeutic target for rare form of melanoma, clinical trial of imatinib + binimetinib for KIT-mutant melanoma	2018	R	Generate of additional models of rare forms of melanoma for their investigation and for screening potential therapies
30017245		PDX residual disease model in mice → New target for therapy (RXRG)		M D	Investigate mechanisms of metastasis and residual disease, and identify targets for overcoming therapeutic resistance
29101162		Organotypic tumor spheroids → New targets for therapy (TBK1 and IKKε)		D I	Resource for modeling dynamic response to immune checkpoint inhibitors, evaluating tumor-immune microenvironment, and developing precision immuno-oncology
29334371		PDX models → Efficacy of AXL-targeted antibody-drug conjugate to treat AXL-high subpopulations in melanoma and other cancers, leading to clinical trials of antibody-drug conjugate			
15611321 12218188 11514604 10430624		Nobel Prize in Medicine for effectiveness of immune checkpoint inhibitor therapy for melanoma discovered with B16/BL6 melanoma cell line and syngeneic mice			
31582381		MITF-low melanoma subtype and residual disease models	2019	D I A	Resource for understanding the MITF-low transcriptional subtype of melanoma and investigating residual disease
30510256		Short wave UV radiation → Conserved signatures in human and mouse		D A	Identify signatures for stratifying patients for therapy
30664638 30188888		Comparative genomic landscapes of human, dog, and horse mucosal melanoma → New drivers of disease		M D R	Understand mechanisms of rare melanoma and BRAF-wildtype melanoma (and veterinary oncology)
31522890		Models of intra-tumoral clonal heterogeneity → Role of neoantigens in response to immune checkpoint inhibitors		M D I A	Evaluate tumor variation and response to treatment to predict biomarkers for stratifying patients for therapy
30940817		RNF5$^{-/-}$ mice → Unfolded protein response linked to gut microbiota and melanoma growth		D I A	Investigate mechanisms linking gut microbiota to anti-tumor immunity
32051401		BRAF^{V600E} GEM melanoma → Discover role for proliferation and stromal remodeling in sustained response to immune checkpoint inhibitors	2020	M D I A	Predict biomarkers for stratifying patients for therapy
32284588		GEM-derived allograft models → Predict immunotherapy response and transcriptional signature in patients			


GEM and GEM-derived allograft
Pros
Immune competent
In vivo imaging
Experimentally robust
Moderate cost
Cons
Non-human context
Low mutation load
WT mice
GEM-derived allograft
GEM
PDX
NSG/nude mice
PDX
Pros
Human relevance
Heterogeneity
High mutation load
Cons
Immune deficient
Xenogeneic TME
High cost
Melanoma subtypes
Cutaneous
Uveal
Mucosal
Acral
Pediatric
2D human cell culture
2D mouse cell culture
2D fish cell culture
Organoids and short-term cultures
Pros
Human relevance
Heterogeneity
High-throughput chemical screens
Experimentally robust
Low cost
Cons
Limited experience with use in melanoma
May lack components of the TME
Zebrafish
Pros
Immune competent or deficient
TME isogenic or xenogeneic
High-throughput chemical and genetic screens
Superior in vivo imaging
Moderate cost
Cons
Non-human context
Low mutation burden