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Melanoma models for the next generation of therapies

E. Elizabeth Patton^{1,*}, Kristen L. Mueller^{2,*}, David J. Adams³, Niroshana Anandasabapathy⁴, Andrew E. Aplin⁵, Corine Bertolotto^{6,36}, Marcus Bosenberg⁷, Craig J. Ceol⁸, Christin E. Burd⁹, Ping Chi^{10,37,38,39}, Meenhard Herlyn¹¹, Sheri L. Holmen¹², Florian A. Karreth¹³, Charles K. Kaufman¹⁴, Shaheen Khan¹⁵, Sebastian Kobold^{16,42}, Eleonora Leucci^{17,43}, Carmit Levy¹⁸, David B. Lombard¹⁹, Amanda W. Lund²⁰, Kerrie L. Marie²¹, Jean-Christophe Marine^{22,40}, Richard Marais²³, Martin McMahon²⁴, Carla Daniela Robles-Espinoza^{25,41}, Ze'ev A. Ronai²⁶, Yardena Samuels²⁷, Maria S. Soengas²⁸, Jessie Villanueva²⁹, Ashani T. Weeraratna³⁰, Richard M. White³¹, Iwei Yeh³², Jiyue Zhu³³, Leonard I. Zon³⁴, Marc S. Hurlbert², Glenn Merlino^{35,*}

¹MRC Human Genetics Unit and Cancer Research UK Edinburgh Centre, MRC Institute of Genetics & Molecular Medicine, The University of Edinburgh, Western General Hospital, Crewe Road, Edinburgh EH4 2XU, UK

²Melanoma Research Alliance, 730 15th Street NW, Washington, DC 20005, USA

³Experimental Cancer Genetics, Wellcome Sanger Institute, Hinxton, Cambridge CB10 1SA, UK

⁴Department of Dermatology, Meyer Cancer Center, Program in Immunology and Microbial Pathogenesis, Weill Cornell Medicine, New York, NY 10026, USA

⁵Department of Cancer Biology, Sidney Kimmel Cancer Center, Thomas Jefferson University, Philadelphia, PA 19107, USA

⁶Université Côte d'Azur, Nice, France

⁷Departments of Dermatology, Pathology, and Immunobiology, Yale University, New Haven, CT, USA

⁸Program in Molecular Medicine and Department of Molecular, Cell and Cancer Biology, University of Massachusetts Medical School, Worcester, MA, USA

⁹Departments of Molecular Genetics, Cancer Biology, and Genetics, The Ohio State University, Biomedical Research Tower, Room 918, 460 W. 12th Avenue, Columbus, OH 43210, USA

¹⁰Human Oncology and Pathogenesis Program, Memorial Sloan Kettering Cancer Center, New York, NY, USA

¹¹The Wistar Institute, Philadelphia, PA, USA

¹²Department of Surgery, University of Utah Health Sciences Center, Huntsman Cancer Institute, University of Utah, Salt Lake City, UT 84112, USA

¹³Department of Molecular Oncology, H. Lee Moffitt Cancer Center and Research Institute, Tampa, FL 33612, USA

*Correspondence: e.patton@igmm.ed.ac.uk (E.E.P.), kmueller@curemelanoma.org (K.L.M.), gmerlino@helix.nih.gov (G.M.).

¹⁴Washington University School of Medicine, Department of Medicine, Division of Oncology, Department of Developmental Biology, McDonnell Science Building, 4518 McKinley Avenue, St. Louis, MO 63110, USA

¹⁵Department of Pathology, UT Southwestern Medical Center, Dallas, TX, USA

¹⁶Center of Integrated Protein Science Munich (CIPS-M) and Division of Clinical Pharmacology, Department of Medicine IV, Klinikum der Universität München, LMU, Munich, Germany

¹⁷Laboratory for RNA Cancer Biology, Department of Oncology, LKI, KU Leuven, 3000 Leuven, Belgium

¹⁸Sackler Faculty of Medicine, Tel Aviv University, Tel Aviv 69978, Israel

¹⁹Department of Pathology, Institute of Gerontology, and Rogel Cancer Center, University of Michigan, Ann Arbor, MI 48109, USA

²⁰Ronald O. Perelman Department of Dermatology and Department of Pathology, NYU Grossman School of Medicine, New York, NY 10016, USA

²¹Laboratory of Cancer Biology and Genetics, Center for Cancer Research, National Cancer Institute, National Institutes of Health, Bethesda, MD 20892, USA

²²Laboratory for Molecular Cancer Biology, Center for Cancer Biology, VIB, Leuven, Belgium

²³CRUK Manchester Institute, The University of Manchester, Alderley Park, Macclesfield SK10 4TG, UK

²⁴Department of Dermatology & Huntsman Cancer Institute, University of Utah, Salt Lake City, UT, USA

²⁵Laboratorio Internacional de Investigación sobre el Genoma Humano, Universidad Nacional Autónoma de México, Campus Juriquilla, Boulevard Juriquilla 3001, Santiago de Querétaro 76230, Mexico

²⁶Cancer Center, Sanford Burnham Medical Discovery Institute, La Jolla, CA 92037, USA

²⁷Department of Molecular Cell Biology, Weizmann Institute of Science, Rehovot, Israel

²⁸Spanish National Cancer Research Centre, 28029 Madrid, Spain

²⁹The Wistar Institute, Molecular and Cellular Oncogenesis Program, Philadelphia, PA, USA

³⁰Department of Biochemistry and Molecular Biology, Johns Hopkins Bloomberg School of Public Health, and Department of Oncology, Sidney Kimmel Cancer Center, Johns Hopkins School of Medicine, Baltimore, MD 21205, USA

³¹Department of Cancer Biology & Genetics and Department of Medicine, Memorial Sloan Kettering Cancer Center, New York, NY, USA

³²Departments of Dermatology and Pathology, University of California, San Francisco, CA, USA

³³Department of Pharmaceutical Sciences, College of Pharmacy and Pharmaceutical Sciences, Washington State University, Spokane, WA, USA

³⁴Stem Cell Program and Division of Hematology/Oncology, Boston Children's Hospital and Dana Farber Cancer Institute, Howard Hughes Medical Institute, Harvard Medical School, Harvard Stem Cell Institute, Stem Cell and Regenerative Biology Department, Harvard University, Boston, MA, USA

³⁵Center for Cancer Research, NCI, NIH, 37 Convent Drive, Bethesda, MD 20892, USA

³⁶INSERM, Biology and Pathologies of Melanocytes, Team 1, Equipe Labellisée Ligue 2020, Centre Méditerranéen de Médecine Moléculaire, Nice, France

³⁷Weill Cornell Graduate School of Medical Sciences, Cornell University, New York, NY, USA

³⁸Department of Medicine, Memorial Sloan Kettering Cancer Center, New York, NY, USA

³⁹Department of Medicine, Weill Cornell Medical College, New York, NY, USA

⁴⁰Laboratory for Molecular Cancer Biology, Department of Oncology, KU Leuven, Leuven, Belgium

⁴¹Wellcome Sanger Institute, Hinxton, Cambridgeshire CB10 1SA, UK

⁴²Member of the German Center for Lung Research (DZL), German Center for Translational Cancer Research (DKTK), partner site Munich, Munich, Germany

⁴³Trace, Department of Oncology, LKI, KU Leuven, 3000 Leuven, Belgium

SUMMARY

There is 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, patient-derived xenograft, 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 preclinical 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 has improved the prognosis for cancer. We have made remarkable progress in the last 10 years, with 13 new melanoma therapies approved in the United States, including both targeted and immune-based therapies (Figure 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. Inter-leukin-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 with 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 to 2016 among people of European ancestry, 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 with 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) preclinical discoveries and insights into therapeutic modalities, and (3) understanding and predicting therapy mechanisms of action and response (Figure 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 (Figure 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 autoimmune disease and rejection of transplanted tumors (Iwai et al., 2002, 2005; Leach et al., 1996; van Elsas et al., 1999, 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).

In this Perspective, 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. Furthermore, we identify necessary advances to improve models, now and in the future, to make breakthroughs in these critical areas.

MELANOMA MODELS

Genetically engineered mouse 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 (Figure 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). Genetically engineered mouse (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 undoubtedly 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. Hepatocyte growth factor-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 inter-follicular cutaneous melanocytes and are useful for studying tanning and melanoma development (Cui et al., 2007; Glover et al., 2015; Kohler et al., 2017).

UV radiation (UVR) exposure is a major risk factor for human melanoma, and the cause of genome-wide mutations (Cancer Genome Atlas Network, 2015). UVR has been incorporated into GEMs to address tanning and burns and 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 the 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 in demonstrating 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 have provided the molecular evidence to support public health melanoma prevention campaigns.

Syngeneic models

Syngeneic transplantation models have long been a workhorse for preclinical 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 anticancer 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) (Figure 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 such as ovalbumin or lymphocytic choriomeningitis virus 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 xenograft models

Patient-derived xenograft (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; Einarsdottir et al., 2014) and can be used to explore the role of metabolism (Tasdogan et al., 2019) and of primate-specific genes, such as long non-coding RNAs in melanoma that 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. (2017) described a collection of 459 PDX models derived from 384 patients. Most of these samples were derived from metastatic lesions, representing all major genetic drivers (e.g., *BRAF*, *NRAS*, *NFI*) 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 preclinical 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., 2016, 2017).

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 proved 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 for other diseases such as colon cancer, limited experience so far exists in utilizing melanoma organoids for drug screens and preclinical 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 three-dimensional (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 have 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 (Figure 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 knock out 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 (transgene electroporation in adult zebrafish) 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, leading 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., 2008, 2011). 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 (Prykhodzhiy 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 that 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 Network, 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 (tumor virus A) 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 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 redesigned 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 Rooijen 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). Furthermore, upon invasion into adipocyte-rich environments, lipid uptake is dependent on the melanoma-expressed fatty acid transporter proteins (FATPs), 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 preconditioning of distant sites to melanoma metastatic niches by the cytokine midkine (Olmeda et al., 2017). This induction at distal premetastatic 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 preconditioned 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. Moreover, while GEM-derived melanoma cells have the same engineered genetic aberrations, heterogeneity can be incorporated by exposing mice to a 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 that 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 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. Furthermore, in 39% of cases resistance was not accounted for by any validated mutations but displayed transcriptomic alterations (Cancer Genome Atlas Network, 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). Furthermore, 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 of 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, 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; Vredevogd et al., 2019; Strub et al., 2018). Exemplifying the importance of metabolism in drug-resistance mechanisms, Bernards and colleagues demonstrated that MAPK-pathway resistance is associated with drug-induced increased levels of reactive oxygen species (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 tumor necrosis factor α (TNF α) to promote melanoma growth; inhibiting TNF α 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 microphthalmia-associated transcription factor (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 toward a differentiated cell state where they are sensitive to a tyrosinase-dependent prodrug (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 subpopulation 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 modeling 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 modeling 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 preconditioned tumor site (Marine et al., 2020). Persister cells represent a small subpopulation of cancer cells that may not necessarily have acquired genetic mutations that drive resistance, and their plasticity can be characterized 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 (RNA-seq) of *BRAF*-mutant PDXs was used to identify four BRAF/MEK inhibitor-tolerant residual subpopulations, with one subpopulation contributing to disease recurrence featuring MTF-low-to-no activity and regulated by RXR- γ (Rambow et al., 2018). RXR- γ 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 microenvironmental 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 in delaying or even eliminating 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, are 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 premetastatic niche. Furthermore, 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 preclinical 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 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 as well as to inform clinical melanoma trials (Perez-Guijarro et al., 2020).

New models are being developed to help understand why many patients are unresponsive to ICI, while others suffer side effects associated with an autoimmune disease yet benefit from sustained long-term cancer suppression (Chamoto et al., 2017; Kumar et al., 2020). New mouse models may avoid foreign or viral antigens, such as ovalbumin and viral antigens, and instead stimulate an 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 preclinical 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-2 γ^{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 proved 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 models with functional mature human lymphocytes represents a major technical challenge but remains a high priority (Forsberg et al., 2019; Jespersen et al., 2017). 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 et al., 2021). 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 (Hölzel and Tüting, 2016). These include, but are not limited to, the (lymph)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 major histocompatibility complex I (MHC I) presentation and perturbed interferon- 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 physiological 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 with younger patients (Kugel et al., 2018), but *in vivo* studies of melanoma tend to use 6- to 8-week-old mice, which corresponds to ~20-year-old humans, and developing/young-adult zebrafish. However, subcutaneous implantation of melanoma tumors into 12- to 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 accurate modeling of human disease, understanding underlying drivers, and identifying 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, Epstein-Barr virus, influenza viruses (Kugel et al., 2018), and possibly even COVID-19, shape the immune repertoire and thus can affect 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 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, UVR plays only a minor role if any, in the etiology of acral, mucosal, and uveal melanoma at sun-

protected sites (Hayward et al., 2017; Kong et al., 2017b; Liang et al., 2017; Robertson et al., 2017; Yeh et al., 2019). However, sun exposure is a risk factor for a subset of ocular melanomas (Johansson et al., 2020b; Karlsson et al., 2020; Mundra et al., 2021). 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 cooperates 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 preclinical testing of palbociclib, a Food and Drug Administration-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. Furthermore, 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 United Kingdom-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 preclinical fish and mouse models, and the impact on melanoma biology studied in the context of a whole

animal system. Predictive preclinical 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 whether age and environmental exposures influence melanogenesis pathways. A simplified melanoma genome in models compared with 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 the capture of transcriptomic, exome, proteomic, chromatin immunoprecipitation-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, 2018; Soldatov et al., 2019; Varum et al., 2019; Zilionis et al., 2019). Evolutionarily conserved signatures/pathways identified in models have proved 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 which data are generated and analyzed will help answer novel questions. For example, single-cell RNA-seq atlases of patient cohorts or melanoma models, generated by collating disparate experiments representing multiple biological contexts, are beneficial for comprehensive analyses. Heterogeneous 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 Network, 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 Network, 2015; Liu et al., 2018; Marie et al., 2020). Other patient datasets are also available that have their own unique setup and data collection, including patient-matched whole-exome and transcriptome sequencing data (Van Allen et al., 2015), pretreatment samples (Hugo et al., 2016; Van Allen et al., 2015), and 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 personalized 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 and 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, 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. (2017) have been provided to a commercial vendor for distribution (Horizon/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/iki/trace/trace-leuven-pdx-platform>) and are 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 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. Furthermore, 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 to eradicate this disease.

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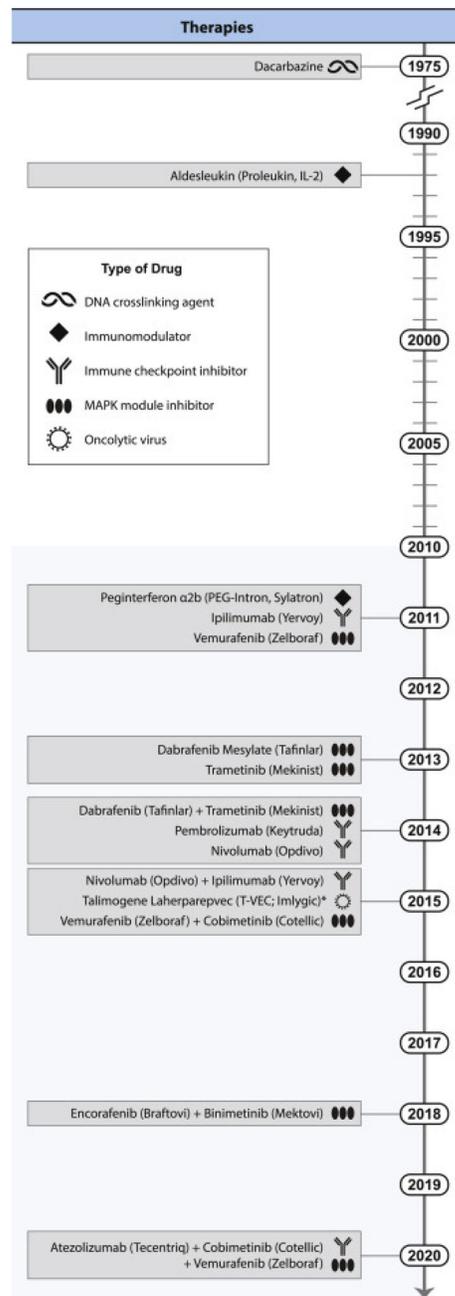


Figure 1. Timeline of treatment approvals by the US Food and Drug Administration for advanced melanoma patients

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.

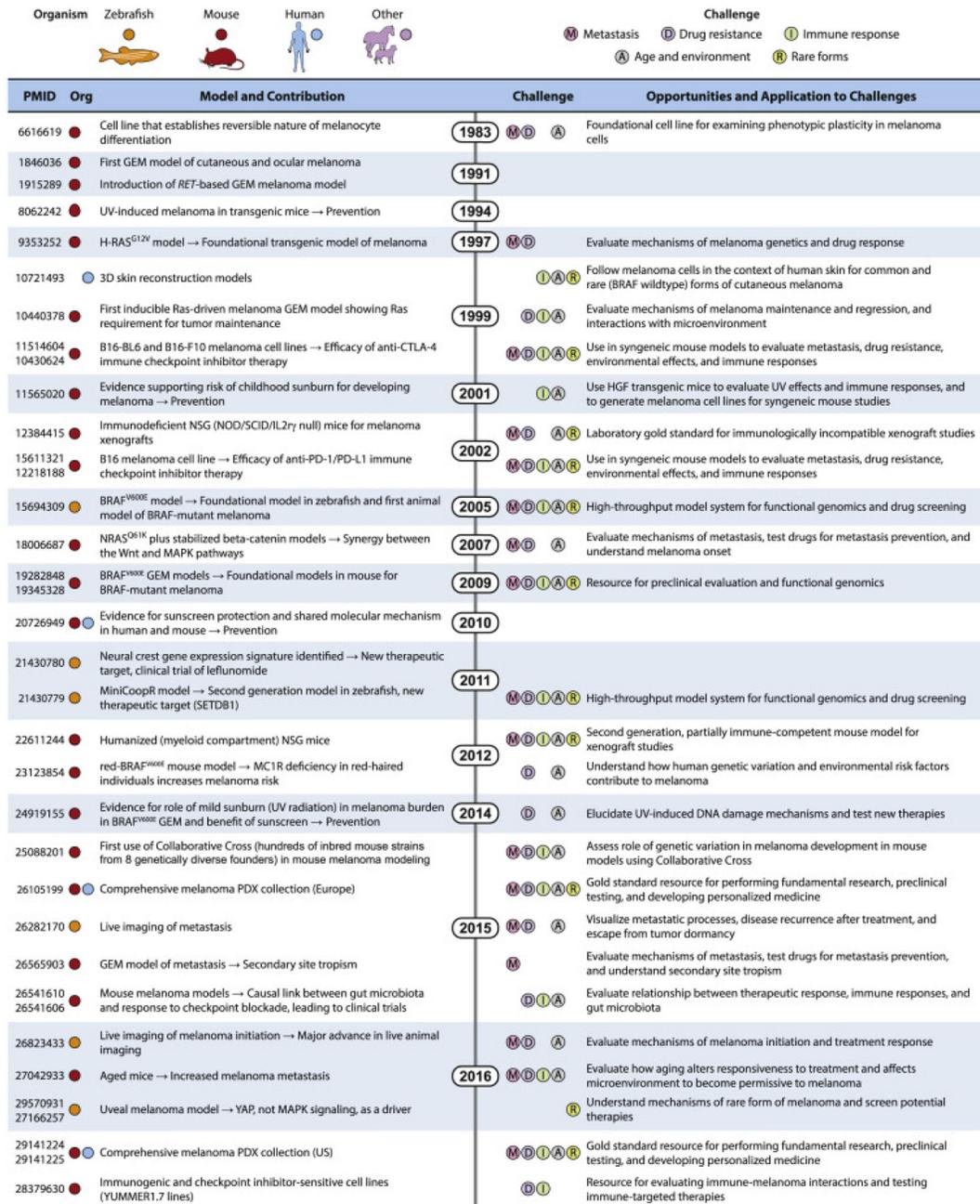


Figure 2. Timeline of melanoma models and opportunities for addressing key challenges
 A brief selection of models from mouse, zebrafish, human, and other species that have made major contributions to our understanding of melanoma biology and in the discovery of new therapies. Such models are critical to address the most pressing challenges in melanoma, including metastasis, drug resistance, immune response, aging 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 are indicated by letters in colored circles. Credit: Heather McDonald, Nancy R. Gough, BioSerendipity.

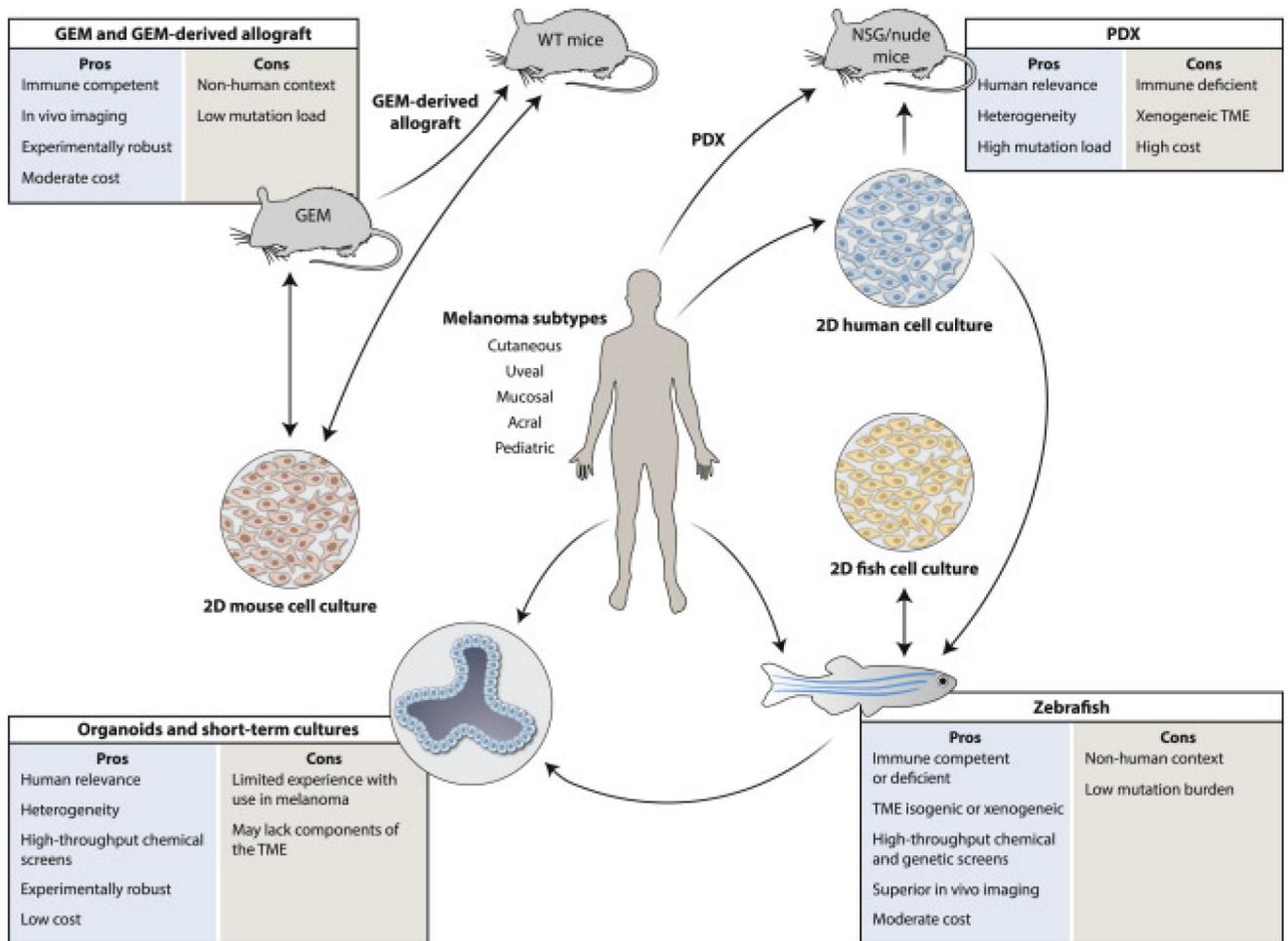


Figure 3. Schematic representation of current melanoma models and the many ways they can be employed to develop new therapies

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 with 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) provides additional layers of experimental possibilities. 2D, two-dimensional; GEM, genetically engineered mouse; GDA, GEM-derived allograft; NSG, NOD/SCID/IL-2 γ ^{null} immunodeficient mouse; PDX, patient-derived xenograft; TME, tumor microenvironment; WT, wild-type.