Published in final edited form as:

Pigment Cell Melanoma Res. 2013 July ; 26(4): E8–E14. doi:10.1111/pcmr.12099.

The Future of Preclinical Mouse Models in Melanoma Treatment Is Now

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On October 17th, 2012 a relatively small group of 28 basic researchers and physician scientists met at the Wistar Institute in Philadelphia, PA to initiate detailed discussions on the current status and future prospects of preclinical studies of human melanoma, focusing on the mouse as a model system. The goal of this meeting, organized by Drs. Glenn Merlino (NCI), Terry Van Dyke (NCI), and Meenhard Herlyn (Wistar), was to facilitate the development of better preclinical melanoma models by giving oncologists the opportunity to tell modelers what information they need in order to improve clinical trials, and have modelers acquaint clinicians with the preclinical state-of-the-art. Participating physician scientists included Drs. Keith Flaherty (Harvard), Lynn Schuchter (U Penn), David Solit (Memorial Sloan Kettering), Suzanne Topalian (Johns Hopkins), Clemens Krepler (Wistar), and Nicolas Acquavella Pesantes (NCI). In addition to the organizers, participating basic researchers included Drs. Andrew Aplin (Thomas Jefferson), Marcus Bosenberg (Yale), Sheri Holmen (Huntsman Cancer Institute), Martin McMahon (UC San Francisco), and Keiran Smalley (Moffitt Cancer Center). Other notable attendees were Drs. Ken Fasman (CSO Adelson Medical Research Foundation), Alison Martin (CMO, Melanoma Research Foundation Breakthrough Consortium), and Wendy Selig (President, CEO Melanoma Research Alliance).

An underlying premise of the Wistar meeting was that no clinical trial should be conducted without a solid rationale derived from predictive preclinical studies. Unfortunately, clinical trials always seem to be one step ahead of the preclinical studies that should be used to support them. Candidate anti-cancer drugs are currently tested in preclinical mouse models that typically employ the growth responses of established human melanoma cell lines subcutaneously xenografted into immunocompromised mice as efficacy endpoints. Such models are overly reliant on cell lines that have been altered through adaptation to growth in culture and have an inadequate tumor microenvironment that includes the lack of an immune system, and so have proven to be poorly predictive of clinical outcome. As a consequence, many drugs move into the clinic without appropriate preclinical support, and most drugs fail. The melanoma field finds itself in dire need of improved preclinical models that are more predictive of clinical response. This report will review meeting highlights, including discussions on the current state of melanoma therapy, on promising mouse melanoma models, and how these two can best be integrated into better clinical care. We also provide a description of new resources that could facilitate preclinical testing, and recount discussions and conclusions concerning immediate clinical priorities and future prospects and challenges.

CLINICAL UPDATE AND PERSPECTIVE

Drs. Flaherty and Topalian began the meeting by bringing the audience up-to-date on the status of targeted and immune-based melanoma therapy, respectively. Significant progress has been made in the treatment of melanoma by focusing on the molecular disease subgroups, as delineated by their driver oncogenes. A driver oncogene can be defined by the consequences of its inhibition: direct targeting of the oncogene product would result in a single-agent anti-tumor effect, be it regression or stasis. For nearly 50% of patients, BRAF^{V600E} plays this role and targeting mutant BRAF or simultaneously inhibiting both BRAF and MEK significantly improves survival in the metastatic setting and may improve cure rates in the adjuvant setting. For an additional 20% of patients mutant NRAS is the driver, but the inability to as-of-yet directly target NRAS has forced the employment of indirect targeting strategies. Single-agent MEK inhibition has a potential role, but a significant minority of patients receive no benefit from this treatment, and those who do have responses that are of less magnitude or duration than BRAF-mutant melanomas treated with BRAF inhibitors. For 1% of patients, c-KIT mutations appear to be the likely drivers, but the mutations are not entirely aligned with available c-KIT inhibitors with regard to molecular or clinical susceptibility. For an additional 1% of all cutaneous melanoma cases (but 80–90% of uveal melanoma cases) GNAQ or GNA11 mutations are the drivers. Unfortunately, as with NRAS, indirect strategies appear to be the only tractable approach in the near term. Notably, in nearly 30% of all melanoma cases the driver oncogene, assuming there is one, has not yet been identified. Preclinical models will be needed to identify which of the recently identified novel melanoma oncogenes, if any, could play this role.

It is almost certain that the most successful future anti-melanoma therapies will consist of combinations of drugs that individually have modest or transient clinical activities. The most successful of combination molecularly targeted therapies to date, a BRAF inhibitor plus a MEK inhibitor in BRAF inhibitor naive patients with mutant tumors, is associated with a 10% complete response rates, 75% objective response rates, median progression free survival of 9–10 months, and one year survival rate of 80%. While these results are very exciting compared to where the field was only 5 years ago, there is a clear need for further advances in this molecular subtype - and the others are far worse. For NRAS mutant melanoma, the best reported outcome with a single-agent MEK inhibitor is no complete responses, 20% objective response rate, and median progression-free survival of 4 months. For c-KIT mutant melanoma, the response rate is 10–20% with median progression-free survival of 4 months. Based on the available preclinical data generated with immortalized melanoma cell lines in vitro and/or in vivo, a number of therapeutic strategies can be justified in genetically defined patient subpopulations (Table 1). The immediate concerns with regard to the clinical translation of these various treatment strategies are: 1) whether there is equally compelling preclinical evidence to support a clinical trial, 2) how to optimally select patients for each and 3) how to anticipate likely resistance mechanisms. For the entire approach of stimulating anti-tumor immunity, patient selection and resistance mechanisms are almost entirely unknown. Finding the answers to all of these critical questions primarily in patients will come at a substantially greater financial and patient cost and require significantly more time than in appropriate animal models.

The melanoma field is currently confronted with the ‘challenge of the riches’ because the pharmaceutical industry is eager to test each new generation of signaling inhibitors in melanoma patients. Sorting out the different inhibitors in patients as to which inhibitor is most optimal or which target specificity hits the melanoma cells strongest with the least side effects will take years and tens of millions of dollars in costs for large clinical trials. Much of the drug comparison could be done in preclinical models, which should also be front-line in assessing whether combination drugs should be given at the same time or in sequence.

Historically, malignant melanoma has been viewed as an immune-responsive disease, and the prospects of treating melanoma by immunotherapy are promising. This is evidenced by the ability of adoptively transferred tumor-infiltrating lymphocytes to mediate durable complete responses in some patients with metastatic melanoma. This notion was recently further validated by the approval of Ipilimumab, an immune-modulatory agent that has paved the way for the development of new strategies that will target both the effector and inhibitory arms of the immune system. As with all human studies, immunotherapy trials for melanoma should be consistently conducted with a solid rationale from preclinical animal efficacy studies. Therefore, the development of clinically relevant, predictive mouse models should be a matter of precedence, as future scientific progress in the field of tumor immunotherapy will also likely depend upon careful development of appropriate preclinical models of melanoma.

PRECLINICAL UPDATE AND PERSPECTIVE

The melanoma field now finds itself with multiple promising candidate models for preclinical testing. These include human and mouse melanoma cell lines, patient-derived xenograft (PDX) models, genetically engineered mouse (GEM) models, and GEM-derived allograft (GDA) models. All have strengths and weaknesses, and all need further validation. Drs. Krepler, Bosenberg, McMahon, Acquavella Pesantes, Holmen and Merlino discussed these various models in some depth. Established human and mouse melanoma cell lines continue to be workhorses for mechanistic studies. Subjecting such lines to drug selection schemes can yield useful information about how cancer cells become drug resistant, as exemplified in Dr. Solit’s work on arising resistance to BRAF^{V600E} inhibition. However, these lines are inexorably altered during adaptation to culture, as described above, limiting their ability to predict drug clinical activity. An approach that is becoming commonplace is the use of melanoma cells that have had their exposure to in vitro culture limited to a very small number of passages. For drug studies the use of fresh PDXs has become particularly attractive because it avoids cell culture altogether.

Human melanoma cells will, even when injected in very small numbers, reliably form a tumor graft in permissible mouse models, irrespective of stage, mutational background, and time in cryogenic storage. PDX samples are collected under IRB approval as fresh biopsy tissue or fine needle aspirate. Within hours tumor chunks, aspirates or purified cell suspensions are implanted subcutaneously in NOD scid IL2 receptor gamma chain knockout (NSG) mice, which lack a functional immune system. Melanoma PDXs take at a 90% rate, and can then be expanded over 3 mouse passages, and stored in a live tumor bank. Dr. Herlyn’s lab has already successfully established a program in which over 140 PDXs from a

wide spectrum of melanoma tissues have been generated and banked, including from patients who have relapsed after BRAF or BRAF/MEK inhibitor therapy. To maintain the same conditions as in patients, the mice are continuously treated with the inhibitors.

Our ability to choose optimal second line single or combination agents is highly dependent on our understanding of the biological signatures of melanomas with intrinsic or acquired resistance to first line agents such as kinase inhibitors. It is anticipated that libraries of PDX models will facilitate this analysis, allowing the identification in populations of biomarkers associated with positive and negative drug responses. But a single PDX can also be used in real time to help the patient from which it was derived. By creating avatars of a patient's melanoma that has relapsed on current therapies or clinical trial protocols, "mini human-in-mouse trials" can be conducted to facilitate the selection of effective drugs, drug combinations and dosing regimens for that specific patient. The often-rapid progression of stage IV melanoma patients makes the latter a challenging goal, but with the development of novel therapies inducing longer remissions and the move of targeted and immuno-therapy to adjuvant setting this might soon be a reality. The preclinical PDX model may represent a significant improvement in the ability to more accurately predict clinical activity. However, the PDX models have limitations, most notably the required use of immunocompromised mice to study human tumors. To effectively study the promising class of immune-modulatory therapies in melanoma, especially in combination with kinase inhibitors, ending the artificial separation of those two fields in pre-clinical research, a fully functioning human immune system would have to be introduced into any tumor graft models. In principle, this can be achieved using humanized mice, in which human CD34+ hematopoietic stem cells introduced into mice subjected to gamma irradiation-induced myeloablation create a functioning human immune system within 3 months. However, this technology is immature and the humanized mice expensive, prompting many investigators to continue to improve GEM models, aiming to make them more amenable to preclinical studies.

Although mice rarely spontaneously develop melanoma, they can be genetically engineered to do so by activating oncogenes relevant to human melanoma, such as mutant BRAF or mutant NRAS, or through inactivation of key tumor suppressors, including CDKN2A and PTEN. The combination of a deeper molecular understanding of melanoma and continual technological advances (e.g., inducible Cre-based recombination and tet-inducible transgene expression) has resulted in the development of mouse models that more closely resemble human melanoma, and will continue to do so. Expression of hepatocyte growth factor/scatter factor ligand for the MET receptor allows melanocytes to survive outside hair follicles, their typical location in mouse skin, to the epidermis, where most reside in human skin. Ultraviolet radiation, thought to play a role in 80% of human melanomas, is also being incorporated into model systems. GEMs can be employed directly as preclinical models, ensuring the analysis of incipient melanoma in the context of a functional immune system.

A new approach to melanoma modeling, the RCAS/TVA system, is being used by Dr. Holmen. In these models, transgenic mice expressing the avian retroviral receptor, TVA, under the control of a melanocyte-specific promoter are susceptible to infection by avian leukosis virus-based retroviral vectors encoding relevant melanoma oncogenes. Infection

results in stable integration of the virus into the genome of replicating cells, allowing for long-term expression. The major advantage of these models is that one transgenic mouse strain can be used to evaluate multiple genes implicated in melanomagenesis either alone or in combination. Moreover, this system permits temporal and spatial control of gene expression, which allows additional studies of tumor progression and maintenance. Tumors evolve from gene mutations in developmentally normal cells in the context of an unaltered microenvironment, which more closely mimics the human disease.

Some advantages of using GEM models can also be disadvantages for preclinical studies. In the best models tumors arise spontaneously, and therefore less predictably and often at multiple sites. These make designing preclinical studies, particularly metastatic disease studies, problematic. A compromise approach that retains the advantages of many of these models uses GEM-derived allografts (GDAs). Here, spontaneously arising mouse melanomas that have been engineered to mimic the genomic and environmental lesions associated with their human counterparts are harvested, and then transplanted and later expanded through orthotopic transplantation onto immunocompetent house mice. The GDA approach combines the tractability for feasible, high-quality drug studies (as with PDX models) with the validity of maintaining compatible tumor-host interactions, including a fully functional immune system (as with GEM models). GDAs are especially amenable to the study of metastatic disease. Considering actual responses of mouse tumors in the metastatic setting instead of tumor growth delay may help narrow the existing “bench to bedside” translational gap. GDAs and GEMs are both invaluable for evaluating immunotherapies and drugs that affect the immune system. Indeed, since the immune system is likely to play a role in the overall response to any given therapy, the presence of the cognate immune system for any model may be paramount in developing accurate human response hypotheses.

For more than five decades, the tumor immunology field has greatly benefited from the development of the poorly immunogenic B16 mouse melanoma model. However, current in-depth knowledge about the complex molecular basis of human melanoma has revealed that this well-established and widely used cell line does not reflect the genetic underpinnings of human melanoma. Instead, preclinical animal models used for efficacy testing of novel immunotherapeutic modalities should be founded on relevant human biology. To help achieve this, the mutational landscape of human melanoma should be modeled on mouse hosts with intact immune systems (e.g., C57BL/6 mice), allowing the evaluation of the effects of the immune system and host-tumor interactions. Ideally, a tumor exhibiting intrinsic immunogenicity on an immunocompetent host would result in the intratumoral recruitment of cellular elements comprising both the innate and adaptive arms of the host immune system. This desired attribute could recapitulate the failure of host immunologic barriers to impede effective antitumor responses in humans.

In addition, the presence of tumor infiltrating lymphocytes in immunocompetent mouse models would recapitulate the occurrence of functionally tolerant T-cell repertoires against unknown tumor antigens in patients with melanoma. A point of concern is the insertion of artificial genetic information (e.g. “non-self” proteins) into tumors that may inadvertently increase their immunogenicity. The artificial aspects of this sort of immunity may not

translate well into human clinical trials compromising the clinical predictive value of the model. Therefore, modeling strategies that circumvent this approach could be prioritized. The major challenge for tumor immunologists is to identify which tumor antigens (e.g., self-antigens, products of mutated genes, cancer testis antigens) are most likely to mediate a successful anti-tumor response. Preclinical modeling of both “self” and “non-self” antigen reactivity is essential for the development of novel immunotherapies. Development of antigenic systems targeting the product of mutated oncogenes and tumor-suppressor genes may serve as definite proof that these epitopes could mediate potent T-cell antitumor responses. This conundrum illustrates the importance of faithfully evaluating antigen-specific immunity and reaching accurate conclusions to appropriately guide the design of clinical trials.

The NCI Center for Advanced Preclinical Research

Dr. Van Dyke is the director of a new preclinical program, the Center for Advanced Preclinical Research (CAPR), developed at the Center for Cancer Research (CCR, NCI). CAPR is a novel resource designed to provide a bridge between basic discovery and applied cancer research in both intramural and extramural communities. CAPR is staffed on a contractual basis from the Scientific Applications International Corporation (SAIC), and has all relevant expertise and technologies to move basic discovery into preclinical assessment at a scale that will inform clinical trial design. Thus, CAPR provides basic and clinical scientists with access to preclinical assessment of potential therapeutics, biomarkers, and novel therapeutic targets while developing “best practices” for preclinical predictability of human studies without draining resources central to the basic and clinical efforts themselves. CAPR proficiencies include: 1) biomarker/molecular signature assessment of disease stages and therapeutic responses, 2) hypothesis generation for clinical research based on systems responses to therapeutics, 3) preclinical evaluation of combinatorial therapies, 4) comparative assessment of predictive power among models (PDX vs. GEM vs. GDA models), 5) technology development to overcome barriers to scale-up and throughput, 6) development and application of clinically relevant therapeutic endpoints (e.g. molecular imaging), 7) therapeutic target discovery and validation using predictive preclinical cancer models, and 8) development of novel engineering strategies, retooling of established models for preclinical research, and establishment of new models for unmet needs.

CAPR achieves its mission to develop an efficient and predictive preclinical arena through internal projects focused on applied preclinical development and via extensive collaborative partnerships (both private and public) focused on therapeutic and biomarker development. Partnering organizations include pharmaceutical, biotechnology and computational science companies, foundations, and public clinical and basic research institutions (including both academic and non-academic affiliations). Expertise within CAPR is focused on the development and application of GEM and GDA cancer models and cultured cell and organ derivatives. Extension to and cross-evaluation of PDX cancer models are carried out via collaborations. CAPR utilizes a wealth of scientific support centers located at the National Laboratory for Cancer Research and operated by SAIC-Frederick, including the Small Animal Imaging Program (SAIP), the Laboratory of Molecular Technologies (LMT), the Pathology and Histology Lab (PHL), the Transgenic/Knockout Production Core, and the

Laboratory of Proteomics and Analytical Technologies (LPAT). Beyond SAIC cores, CAPR has established interactions with the SAIC-Nanotechnology Characterization Lab (NCL), NIH Chemical Genomics Center (for HTP cell-based screens), the NCI Core Genotyping Facility (CGF), CTEP, DTP, NCI Informational Sciences, and the Mouse Models of Human Cancers Consortium (MMHCC). Current studies include model development, therapeutic assessment and biomarker development in lung adenocarcinoma and squamous cell carcinoma, ovarian cancer, glioblastoma, pancreatic cancer, and melanoma.

MEETING CONCLUSIONS

Melanoma therapy has progressed in the last three years at an unprecedented pace culminating in true benefits for patients not just for stabilizing the disease and prolonged progression-free survival but also for overall survival. The advances were achieved independently with two very different approaches: first, through the immune system, either by passive immunotherapy with patients' T lymphocytes or by blocking negative regulatory molecules on lymphocytes; and second, by blocking signaling molecules with small molecule inhibitors. While strategies for the very next clinical trials appear obvious from a wealth of existing clinical and preclinical data (see Table 1), the future in melanoma therapy beyond the next two years is less clear, particularly in signaling therapy. Guidance for the best future targets and therapies have to come from preclinical studies in which not a single model is utilized, but instead multiple models that synergize to mimic the clinical pathologies and challenges. We need to achieve a high level of coordination between experimentalists and clinicians to ensure that the most appropriate models are used for each disease sub-group. In the recent past, genetic signatures of melanomas have guided strategies for therapy. Most recent genome-wide screens are providing us with a wealth of potentially new targets that will all require validation in experimental models. In addition, novel inhibitors need to come not just from pharma but also academia as they face their many obstacles when moved from a lead compound to a 'real' drug. The better we coordinate our activities, including with pharma, the faster we can move forward. The recent creating of CAPR could be a real boost for the field but we need to find the funding for it, which is a major challenge in the current climate of tight budgets.

Melanoma is a complex disease and genetic events are only one aspect in understanding the disease and for developing new therapeutic strategies. We need to develop biological signatures that encompass the many epigenetic events and microenvironmental signals so critical for this disease. No single laboratory can cope with these multitude of challenges. Instead, we need to develop platforms for communication and exchange of data and models to continue the rapid progress of the last few years. A consortium-type platform with regular meetings and a Steering Committee for coordination should bring the clinical and experimental investigators together. Curing the many forms of melanoma is our challenge and our mission. Only by working together can we achieve our lofty goal.

RECOMMENDATIONS

1. Immediate Clinical Priorities

- Explain the heterogeneity of melanoma patient response (in both scale and duration) to targeted therapy
- Identify mechanisms of primary (intrinsic) and secondary (acquired) resistance to targeted therapies
- Explain why only some patient responses to immunologics are durable
- Identify patient cohorts that will initially respond to a specific immunotherapy
- Identify patient cohorts that will experience untoward side effects/toxicity
- Identify optimal combination therapies and sequences (e.g., targeted, targeted plus immunotherapy, vaccine plus immunotherapy, etc.), and establish optimal sequence of combinations
- Identify new targets/therapies for use when current drug trials plateau next year

2. Longer-Term Goals

- Generate preclinical models that recapitulate melanoma evolving in a clinically relevant context
- Define the optimal predictive range/relevance of each particular preclinical model
- Standardize preclinical studies to allow comparison between models and studies
- Establish a Steering Committee of clinicians and modelers to guide preclinical studies and coordinate collaborations with pharmaceutical companies.

3. Current and Future Challenges

- Modeling heterogeneity and resistance in tumor response to targeted and immunebased therapies
- Identifying biomarkers of therapeutic response/resistance
- Modeling genomic instability in mouse melanoma models
- Humanizing the mouse to allow use of human melanoma in relatively immunocompetent mice
- Understanding the role of metabolism in mouse and human melanomas
- Streamlining the search for new effective agents to replenish the limited pipeline and request for new compounds for preclinical/clinical studies.
- Streamlining functional analyses of candidate targets from genomic studies
- Availability and cost of adequate amount of drugs for preclinical studies

- Combined use of compounds from different pharmaceutical companies

TABLE 1**Proposed Drug and Drug Combination Treatments for Melanoma Molecular Subtypes**

BRAF mutant melanoma	<ul style="list-style-type: none"> • Intermittent administration of BRAF inhibitor • Combination of BRAFi with CDK4 inhibitor • Combination of BRAFi with PI3K/AKT/mTOR inhibitor • Combination of BRAFi with mdm2 antagonist in p53 wt tumors • Combination of BRAFi with HGF/MET inhibitors • Combination of BRAFi with DHODH inhibitors • Combination of BRAFi T cell-directed immune therapies CTLA-4 inhibitor; PD-1/PD-L1 inhibitor; IL-2
NRAS mutant melanoma	<ul style="list-style-type: none"> • Combination of MEK inhibitor with CDK4 inhibitor • Combination of MEK inhibitor with mdm2 antagonist in p53 wt
GNAQ/GNA11 mutant melanoma	<ul style="list-style-type: none"> • PKC inhibitor • Combination of MEK inhibitor with PI3K/AKT/mTOR inhibitors

For treatment of patients with BRAF mutant melanoma, all drug combinations with BRAFi could also include inhibitors of MEK, which are currently being used in the clinic.