Dual Recombinase–Based Mouse Models Help Decipher Cancer Biology and Targets for Therapy

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ABSTRACT

The advent of next-generation sequencing (NGS) and single-cell profiling technologies has revealed the complex and heterogenous ecosystem of human tumors under steady-state and therapeutic perturbation. Breakthroughs in the development of genetically engineered mouse models (GEMM) of human cancers that are based on the combination of two site-specific recombinase systems [dual-recombinase system (DRS)] offer fundamental new possibilities to elucidate and understand critical drivers of the diverse tumor phenotypes and validate potential targets for therapy. Here, we discuss opportunities DRS-based cancer GEMMs offer to model, trace, manipulate, and functionally investigate established cancers, their interactions with the host, and their response to therapy.

Introduction

Genetically engineered mouse models (GEMM) are crucial to study complex biological processes, such as cancer (1). They have been invaluable in providing fundamental insights into the molecular and cellular basis of malignancies, such as the genetic and epigenetic alterations that drive tumor initiation, progression and metastatic spread to distant sites (1). Such models have also been vital for a better understanding of the complex interactions between cancer cells and their surrounding tumor microenvironment (TME), including the immune system. They highlighted for example the importance of the tumor stroma for tumor progression, immune evasion and drug resistance. Furthermore, GEMMs have been successfully used as preclinical platforms to validate candidate therapeutic targets, test the efficacy of anticancer drugs, and elucidate mechanisms of therapy resistance (1).

In these models, tumorigenesis is typically initiated through the activation of oncogenes and/or the inactivation of tumor suppressor genes *in vivo* by utilizing site specific recombination (SSR) systems, such as Cre/loxP, which allows precise cell type specific tumor induction (1). Consequently, tumors develop *de novo* and evolve in a native immune proficient microenvironment at the site of origin of the respective cancer type in a complex living organism. So far, several GEMMs have been created that faithfully recapitulate the histologic, molecular, genetic, and clinical hallmarks of their human counterparts, including their inter and intratumor heterogeneity, complex stroma composition and spontaneous metastasis formation. As such, these models are indispensable for preclinical research and became one of the gold standards for the investigation of novel diagnostics, drug targets and therapeutic regimes (1).

Altough the establishment of SSR-based cancer models have provided a wealth of information about the biologic basis of cancer, and have helped to develop improved cancer diagnostics and more effective therapies (1), these GEMMs also have some important limitations. They rely on a single-recombination step (e.g., Cre/loxP) to activate oncogenes and/or inactivate tumor suppressors. This has the significant drawback that it does not allow the precise genetic modeling and manipulation of sequential multistep tumorigenesis and tumor heterogeneity, both of which are important hallmarks of the human disease. Such an approach also makes it challenging to validate candidate targets genetically to block progression of precursor lesions, or target established cancers. In addition, evaluation of resistance mechanisms and manipulation of the TME at the genetic level is almost impossible to perform using single SSR systems, such as Cre/loxP. Thus, there is a high need for a next-generation of enhanced preclinical model systems that overcome these limitations (2, 3).

Dual Recombinase-Based Cancer Models

Recent advances in genetic engineering and the development of several different orthogonal SSR systems, such as Cre/loxP, Flp/FRT, Dre/rox, and Vika/vox, has opened up fundamental new possibilities for more precise cancer modeling and complex intersectional lineage tracing and gene manipulation, when used in combination as dual recombinase systems (DRS) in the same organism (1–5). Specifically, the second SSR provides another layer of controlled complexity, as it allows to manipulate distinct genes and cell types at a distinct time point independent of the first SSR. The application of DRS is currently radically changing our ability to systematically investigate the complex ecosystem of cancer cells and their corresponding microenvironment in a realistic manner in space and time at the organismal level *in vivo* (2, 3, 5–7). Access to these aspects is vital for identifying and assessing candidate therapeutic targets and mechanisms of resistance, as well as revealing basic principles of tumor biology.

DRS-based models enable precise spatial and temporal regulation of gene expression *in vivo* through expression of the two recombinases

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Cancer Res 2023;83:2279–82

doi: 10.1158/0008-5472.CAN-22-2119

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under the control of distinct promoters, which can be ubiquitously active (e.g., Rosa26), tissue specific (e.g., insulin for pancreatic beta cells), active in certain phases of development or cell differentiation (e.g., transcription factors regulating epithelial-to-mesenchymal transition; EMT), or in adult animals (e.g., hormones or hormone receptors). When expressed, the recombinases, such as Flp, Cre, Dre, or Vika, can enzymatically recombine their specific recognition sites, distinct short DNA sequences termed *FRT*, *loxP*, *rox*, or *vox* sites, respectively. The result depends on the orientation and position of the two DNA sites, namely, the same orientation leads to excision of the DNA in between, the opposite orientation to inversion of the region, while the site positioning on different DNA molecules results in translocation. Furthermore, by expressing a recombinase under the control of an inducible promoter, such as CYP1A1, which is transcriptionally up-regulated in response to β-napthanoflavone, or by fusing a recombinase with certain receptor ligand-binding domains, such as a mutant estrogen receptor (ER^{T2}), the expression of the recombinase can be induced, or the enzymatic activity of the recombinase can be activated through binding of the ligand, e.g., by tamoxifen-induced nuclear translocation of $CreER^{T2}$, respectively. Depending on the chosen promoters, recombinases, and recombination sites, DRS-based models can be utilized for cell (sub)type specific intersectional activation or deletion of selected genes, multicolor fluorescent protein reporter and lineage tracing approaches, regulated overexpression of genes or additional SSRs, as well as ablation of specific cell population (3, 4, 6, 7).

In vivo cancer models that are based on the DRS have rapidly evolved in recent years and are now widely used in basic and translational research. Though initially applied in sarcoma and pancreatic cancer models (2, 3), the promoter selection of SSR systems enables targeting of various tissues of choice, and has broadened the repertoire to DRS models to breast and lung cancers, glioma and pituitary adenoma. Here, we will discuss the unique opportunities the DRS provides to model, manipulate and functionally investigate tumor biology and response to therapy in whole animals *in vivo*. This includes lineage tracing and rigorous genetic analysis of (i) multistep carcinogenesis and tumor heterogeneity, (ii) the role and function of tumor and host cell subpopulations, such as tumor initiating cells, or fibroblast and immune cell subtypes in the TME as well as their communication and interaction, (iii) EMT and the metastatic process, and (iv) therapeutic targets and resistance mechanisms in autochthonous tumors. Further, we will explore current limitations of DRS models and future directions, which should be pursued to refine them and overcome these limitations. These efforts will lead to the design of further improved multi-recombinase based cancer models with increased flexibility that develop tumors, which closely recapitulate their human counterparts.

Modeling Multistep Carcinogenesis, Punctuated Genetic Events, and Tumor Heterogeneity

The advent of next-generation sequencing (NGS) technologies has revealed distinct routes and modes of tumor evolution. In addition to classical multistep carcinogenesis with the sequential activation of oncogenes and loss of tumor suppressors, it has been shown that alterations of mismatch repair genes or the proofreading machinery, epigenetic mechanisms, including DNA methylation, chromatin remodeling, and histone modifications, as well as single catastrophic genetic events, such as whole-genome doubling, chromothripsis, and chromoplexy are important drivers of tumor evolution, which strongly impact clinical phenotypes and therapeutic vulnerabilities. Therefore, it is of fundamental importance to model and mechanistically understand the different routes and modes of tumor evolution at the organismal level.

Multistep carcinogenesis and catastrophic genetic events

DRS-based cancer models offer unique opportunities to investigate the cooperation and mutual exclusivity of genetic events in space and time (i.e., stepwise vs. single-punctuated catastrophic events) thereby opening the possibility to mechanistically study for example epistasis in cancer. The stepwise activation of an oncogene and the time-controlled inactivation of a tumor suppressor gene has been demonstrated recently in a DRS-based mouse models of KRAS-driven pancreatic cancer. Using a *Pdx1-Flp* allele to induce oncogenic KRAS and $CreER^{T2}$ expression in the murine pancreas combined with the subsequent time controlled inactivation of a floxed *Trp53* allele, the critical role of p53 in controlling the progression of premalignant pancreatic intraepithelial neoplasia (PanIN) to invasive pancreatic ductal adenocarcinoma (PDAC) has been demonstrated (3). Furthermore, the DRS offers possibilities to delete or activate multiple genes in parallel in an inducible manner (e.g., by tamoxifen mediated $CreER^{T2}$ activation), thereby modeling catastrophic genetic events that result in the simultaneous acquisition of multiple strong cancer drivers at a given timepoint. Further, low-dose tamoxifen treatment can be used to delete and/or activate the expression of multiple genes in a mosaic fashion, which allows the investigation of genetic interactions and their cooperation in mediating e.g., fitness advantages *in vivo* or to study haploinsufficient phenotypes.

Tumor heterogeneity

Intratumoral heterogeneity is a hallmark of almost all solid cancers and a major driver of their aggressiveness and therapy resistance. Most tumors evolve under evolutionary pressure, resulting in a considerable degree of genetic, epigenetic, and phenotypic heterogeneity. As described above, the DRS offers the possibility to generate mosaic tumors, which harbor incompletely and completely recombined alleles, thereby creating tumors with various combinations of defined genetic modifications, which can be used to model intratumoral heterogeneity. Moreover, the DRS allows for controlled initiation of mosaic tumors and in parallel lineage tracing of the resulting subclones. Mosaic analysis by dual recombinase-mediated cassette exchange (MADR) as well as mosaic analysis with double markers (MADM) represent novel systems of introducing defined mutations and lineage trace the resulting subclonal populations, which express/retain or lack specific cancer drivers, or tumor suppressors, such as p53. MADR enables for example the engineering of gliomas in mice, which recapitulate the mutational and transcriptional heterogeneity of their human counterparts (8). Thus, the system offers the possibility of personalized modeling and drug testing on a wide array of driver mutations and their combinations.

Dissecting the Functional Role of Cancer and TME Cell Subpopulations and Their Interaction and Communication

Normal tissue architecture is drastically changed during tumor development and progression. Besides the changes in the cancer cells,

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the surrounding environment is shaped by complex interactions between various different cell types, including subclonal cancer populations, immune cell subsets, and different stromal cell types (e.g., fibroblast subtypes, endothelial cells, or nerves). It has been shown that the TME is a critical driver of tumor evolution, intratumoral heterogeneity and therapeutic response and resistance; however, the specific genetic manipulation and therapeutic validation of cancer and TME cell subpopulations and the investigation of their interactions remained challenging (9).

The DRS offers unique avenues to address these limitations and decipher the impact of cancer and TME cell organization and composition on tumor phenotypes. By combining the Flp/FRT and the Cre/loxP system, cancer cell-autonomous as well as non-cell-autonomous mechanisms of tumor initiation and progression have been uncovered. Combining for example Flp/FRT-based oncogene activation with stromal or immune cell specific Cre driver lines, the DRS allows to genetically manipulate specific TME cell types and dissect their role in tumor development and progression (3, 9). In DRS-based models of PDAC, NOTCH signalling has been uncovered as a critical mediator of tumor-associated macrophage abundance and polarization. Activation of the Notch pathway in macrophages resulted in a decrease of protumorigenic M2-like macrophages and increased survival. Mast cell depletion by utilizing the DRS in combination with a mast-cell-specific Cre driver line, revealed that this cell type is dispensable for PDAC initiation (3, 9). Recent evidence suggests that divergent subpopulations of fibroblasts might have distinct functions in tumor progression, acting either as tumor-promoting or -restraining determinants. αSMA^+ myofibroblasts are the main source of collagen I production in the desmoplastic PDAC stroma. Using a Cre driver line under the control of the *αSMA* promoter to delete *Col1a1* in myofibroblasts to block collagen I production accelerated PDAC progression via the recruitment of immunosuppressive myeloid-derived suppressor cells (7). In contrast, deletion of *Col1a1* in pancreatic cancer cells increased T-cell infiltration and overall survival of PDAC bearing animals, which was associated with the reprograming of the tumor microbiome and enabled anti—programmed cell death 1 (PD-1) immunotherapy (6). These studies exemplify how DRS-based models can be employed to specifically target and genetically manipulate selected cell (sub)populations to uncover context-specific mechanisms of tumor progression and identify potential targets for therapeutic intervention.

EMT and Metastasis Formation

EMT has been closely linked to metastasis formation, as it grants tumor cells the ability to migrate and invade the surrounding tissue and access the circulation. However, the process of EMT is dynamic and reversible, thereby increasing the difficulty in studying its exact role in metastasis formation. By using a DRS-based model of PDAC initiation in combination with *αSMA-Cre* or *Fsp1-Cre* driver lines, which recombine cells undergoing partial and full EMT, and a dual fluorescence Flp/Cre dependent reporter line ($R26^{Dual}$), intersectional lineage tracing of the metastatic process became possible (4). Fluorescence reporter gene expression of cells in autochthonous primary tumors and their metastases revealed that only a small fraction of Cre recombined PDAC cells contribute to metastasis formation, indicating that cells with a preserved epithelial phenotype are capable of spreading to distant sites (4).

Identification and Validation of Therapeutic Targets and Elucidation of Therapy Response and Resistance

DRS-based cancer models have been used successfully to genetically validate candidate drugable targets and target combinations, as well as assessing therapeutic efficacy and mechanisms of therapy resistance in autochthonous tumors *in vivo* (3, 5, 9, 10). Importantly, tumors develop in animals with an intact immune system enabling studies that focus on exploring novel immunotherapies, or modulating tumor immune cell interactions (1, 10). The impact of oncogenes and oncogenic signaling pathways on tumor progression and maintenance can be assessed using inducible DRS-based models, in which the respective genes can be permanently deleted in established tumors (1, 9). For example, inducible deletion of the drugable PI3K-downstream signaling node *Pdkp1* resulted in the eradication of PanIN lesions, demonstrating that PanIN-to-PDAC progression depends on active PI3K–PDPK1 signaling (3). Whether PDPK1 is also a target for therapy of established PDAC remains elusive. To investigate the role of the RAF/MEK/ERK signaling pathway in full blown PDAC *in vivo*, floxed *Mek1* and *Mek2* alleles have been genetically inactivated in established cancers *in vivo* by tamoxifen-dependent $CreER^{T2}$ activation, resulting in a delay in tumor progression, but not complete tumor eradication. This indicates that even the complete genetic disruption of the MAPK pathway is not sufficient to treat PDAC efficiently. Subsequent combinatorial drug screens with the MEK inhibitor trametinib as backbone identified efficient combination therapies, which induced PDAC cell death and reprogrammed its immunosuppressive TME thereby sensitising the tumors to immune checkpoint blockade (10). In addition to investigating tumor cell-autonomous pathways for therapeutic interventions, the DRS can be utilized to dissect the role of distinct genes and therapeutic targets in different cell types, i.e., tumor cell (sub) populations versus cells of the TME. For instance, the DRS technology has been used in autochthonous mouse models of sarcoma and lung cancer to test the role of ATM in tumor versus endothelial cells for the sensitization of established tumors towards high-dose radiotherapy. The selective deletion of *Atm* in either of the two cell types revealed that ATM expression in tumor cells rather than endothelial cells is the critical determinant of therapy response to high-dose radiotherapy in both cancer types (5).

Current Limitations and Future Prospects

Although the DRS provides an important upgrade of the zoo of GEM cancer models and has proven extremely useful in preclinical and translational cancer research, there still exist certain limitations and obstacles. Notably, the chosen SSRs as well as the locus and structure of their target sequences significantly impacts on the recombination efficiency. Another critical factor is the choice of the SSR-driving promoter, as promoters can be active to different levels in various tissues throughout the development or might become unexpectedly active in pathologic states, leading to off-target effects, which might confound the obtained results. Nevertheless, some of these limitations can be overcome by DRS-based intersectional gene targeting using different promoters, or by dimerizable SSRs. When using ligand-inducible recombinases, toxicity of the ligand might occur, especially in the developing embryo. Furthermore, cell toxicities of SSRs have been described, especially when the SSR is expressed at high levels.

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The efficiency of recombination is often lower with ligand-inducible in comparison to constitutive systems, which might result in incomplete recombination of the target sequences. Furthermore, temporal control of SSR activity is delayed by the time required for target cell penetration. In addition to ligand-inducible systems, optogenetic light-activatable systems have been developed recently, which can provide a precise and robust mean of spatiotemporal control of SSR activity. They are based on genetically encoded dimerization domains of plant photoreceptors, which regulate protein–protein interactions by light. The activity of a SSR split into two proteins can be activated by such dimerizers, which bring together the two inactive fragments to reconstitute the functional protein, thereby allowing light-dependent control of DNA recombination. However, such systems are restricted by the limited light-penetrance in deep tissues. The multiplexing and inducibility of recombinases can be expanded also by newly engineered split-recombinase systems that can be activated with small molecules or temperature. Another approach is to combine more than two orthogonal SSRs in one organism. Using three or more recombinases in an *in vivo* cancer model would allow for example the targeting of cell types, which are not characterized by a single marker gene, such as subsets of specialized immune cells or fibroblasts (9). One important limitation, especially in the case of more than two recombinase systems, is however the time and effort needed to breed cancer models harbouring multiple alleles. Recently developed somatic DRS-based cancer models and the delivery of the recombinase via e.g., viral gene transfer or electroporation allow fast-track model generation and the fine-tuning of GEM cancer models. In addition to mice, DRS-based strategies can be applied to other species, such as pigs to generate improved large animal models of human cancer, which can be used to test multimodal therapeutic interventions.

Taken together, DRS-based models opened new avenues to the cancer research field to address questions that could not be addressed before. These models have provided pivotal new insights into critical aspects of tumor biology as well as translational oncology. The advent of a multitude of novel technologies and tools, such as CRISPR/Cas and base-editor directed genome engineering in combination with barcoded single cell approaches and novel technologies to inducibly activate or delete genes with high precision in space and time will allow further refinement and fine tuning of DRS-based approaches. They are of high importance to further improve our ability to better understand the extremely complex mechanisms underlying cancer biology, and are essential to improve the translatability of preclinical findings to the clinic.

Authors' Disclosures

C. Falcomatà reports a grant from the Cancer Research Institute and D. Saur reports grants from Deutsche Forschungsgemeinschaft, German Cancer Consortium (DKTK), European Research Council, and Wilhelm Sander-Stiftung during the conduct of the study. No disclosures were reported by the other author.

Acknowledgments

The authors apologize to all colleagues whose primary work was not included in this review article due to space restrictions and the limit of 10 references per article style. This work is supported by grants from the European Research Council (ERC CoG No. 648521), the German Cancer Consortium (DKTK) and DKTK Joint Funding, Deutsche Forschungsgemeinschaft (SFB 1321 Project-ID 329628492 P06; SFB 1371 Project-ID 395357507 P12; DFG ME 4766/2–1 and SA 1374/8–1 Project-ID 515991405; DFG SA 1374/7–1 Project-ID 515571394; DFG SCHO 1732/2–1 Project-ID 360394750; DFG SA 1374/6–1 Project-ID 458890590; DFG SA 1374/4–3 Project ID 219542602), and the Wilhelm Sander-Stiftung (2020.174.1 and 2017.091.2). C. Falcomatà is a Cancer Research Institute Irvington Fellow supported by the Cancer Research Institute (CRI4641).

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Received January 21, 2023; revised April 29, 2023; accepted May 23, 2023; published first July 14, 2023.

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