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REVIEW ARTICLE

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Experimental identification of cancer driver alterations in the era of pan-cancer genomics

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Abstract

Rapidly accumulating data from large-scale cancer genomics studies have been generating important information about genes and their somatic alterations underlying cell transformation, cancer onset and tumor progression. However, these events are usually defined by using computational techniques, whereas the understanding of their actual functional roles and impact typically warrants validation by experimental means. Critical information has been obtained from targeted genetic perturbation (gene knockout) studies conducted in animals, yet these investigations are cost-prohibitive and time-consuming. In addition, the 3R principles (replacement, reduction, refinement) have been set in place to reduce animal use burden and are increasingly observed in many areas of biomedical research. Consequently, the focus has shifted to new designs of innovative cell-based experimental models of cell immortalization and transformation in which the critical cancer driver events can be introduced by mutagenic insult and studied functionally, at the level of critical phenotypic readouts. From these efforts, primary cell-based selective barrier-bypass models of cell immortalization have emerged as an attractive system that allows studies of the functional relevance of acquired mutations as well as their role as candidate cancer driver events. In this review, we provide an overview of various experimental systems linking carcinogen exposure-driven cell transformation with the study of cancer driver events. We further describe the advantages and disadvantages of the currently available cell-based models while outlining future directions for in vitro modeling and functional testing of cancer driver events.

KEYWORDS
bioinformatics, carcinogenesis, epigenetics, experimental cell culture systems, genomic analysis

Abbreviations: 5mC, 5-methylcytosine; AIG, anchorage-independent growth; B[a]P, benzo[*a*]pyrene; BAF, BRG1-associated factor (complex); BBCE, barrier bypass-clonal expansion; BPDE, benzo[*a*]pyrene diol epoxide/benzo[*a*]pyrene-7, 8-dihydrodiol-9, 10-oxide; CGC, Cancer Gene Census; COSMIC, Catalogue Of Somatic Mutations In Cancer; CRISPR, clustered regularly interspaced short palindromic repeats; HBEC, human bronchial epithelial cells; HMEC, human mammary epithelial cells; Hupki, human p53 knock-in; MEF, mouse embryonic fibroblasts; MNU, methylnitrosourea; NCG, Network of Cancer Genes; NGS, next-generation sequencing; SBS, single-base substitution; scsCE, single-cell subcloning-clonal expansion; TCGA, The Cancer Genome Atlas; TP53, official symbol for human p53 (also used for Hupki MEF, which have the human version); Tp53, official symbol for Syrian hamster p53; Trp53, official symbol for mouse p53; VUS, variants of unknown significance; WES, whole-exome sequencing.

1 | INTRODUCTION

As a result of exposure to exogenous carcinogens and/or the effects of endogenous factors, cancer genomes often harbor complex mutation profiles. Most of the changes found in tumors are passenger mutations that accumulate during tumorigenesis but do not critically affect cell fitness. However, a small subset of alterations – cancer driver mutations – confer a selective growth advantage on a cell, which can subsequently lead to the expansion of a clonal cell population and tumor development. A dozen years into the NGS revolution, the scientific community is producing new information on cancer-associated alterations at an unprecedented rate. Lists of genes with putative causal roles in cancer development are being continuously updated and revised. Given the extent and nature of NGS data, the discovery of most of these novel cancer genes and mutations is based on computational approaches that evaluate either mutations in individual genes with respect to background mutation rates[1,2] or the non-random distribution of genetic alterations within biological pathways and networks.[3,4] By applying these strategies, more than 700 genes have been included in the CGC to date.[5] Depending on the data curation approach used, the number of putative cancer driver genes can, however, vary considerably. Based on the CGC data and a manually curated cancer driver list,[6] combined with data from more than 270 cancer sequencing studies, the NCG contains almost 2400 established or putative driver genes.[7] In contrast, a comprehensive analysis of all datasets of TCGA, using stringent sample inclusion criteria and various driver identification tools based on different algorithms, resulted in a list of 299 cancer driver genes.[8] Work by Bailey et al led to the following observations. First, comprehensive analysis confirmed 70%-80% of previously published driver genes from the same datasets; second, among the 299 driver genes, 59 were novel drivers and had not been reported as such before.[8] These findings clearly showed that variation in sequencing data quality, analysis and curation approaches can lead to considerable differences in driver gene identification.

At the moment, an important challenge lies in experimentally validating the role of computationally identified genes and mutations in cell transformation and cancer development. Gene knockout studies in animals and identification of candidate driver gene mutations in animal models of carcinogen-induced tumorigenesis can provide critical functional information. Interestingly, mouse lung tumors induced either genetically (Kras activation) or by exposure to genotoxic carcinogens showed the same activating *Kras* mutations. In contrast to genetically induced tumors, however, chemically induced cancers showed large numbers of SBS in known and putative cancer driver genes, suggesting that carcinogen exposure-based models may provide valuable insight into the complex mutation profiles observed in human cancer.[9] Correspondingly, a handful of studies combining carcinogen-induced tumorigenesis with WES revealed a number of known driver alterations in skin cancer (eg, *Hras*, *Kras*, Notch 1 and *3*, *Trp53*),[10] malignant mesothelioma (eg, *Cdkn2a/p16*, *Trp53*, *Myc*)[11] and liver tumors (eg, *Hras*, *Braf*, *Egfr*, *Apc*),[12] along with a substantial number of putative driver gene mutations. Despite their undisputed significance, animal studies are cost-prohibitive and time-consuming, and they are therefore frequently complemented by experimental work in cellular models of immortalization and transformation. In this context, cell types that rely on the bypass of a biological barrier, such as senescence, can provide valuable information regarding the functional relevance of the identified alterations. Cell models derived from rodents have been especially suitable due to their inherently long telomeres which favor an immortalization process that is driven by alterations in oncogenes and tumor suppressor genes,[13,14] but similar experiments have also been carried out in human primary cells immortalized by exposure to various carcinogens.[15-17] Support for the relevance of using clonal carcinogen-induced immortalization of normal cells as a surrogate for carcinogenesis and a means for driver assessment comes from recent work in normal tissue that shows the presence of driver gene mutations and clonal expansion steps before the onset of any phenotypic signs of tumorigenesis.[18-21] Cellular model systems provide a valuable resource to support the functional impact of alterations identified in (epi)genomic studies of human tumors, and they may be used to assess the functional relevance of these events during early stages of cell transformation.

2 | DRIVER ALTERATIONS IN CELLULAR MODELS OF CARCINOGEN EXPOSURE AND TRANSFORMATION

Immortalization of MEF is, to a large extent, controlled by the p53/p19ARF pathway. Alterations in the mouse *Trp53* gene are commonly found upon spontaneous immortalization of primary MEF[22] and, similarly, selective inactivation of *p19Arf* favors the outgrowth of clones of immortalized MEF.[23] Complementary studies, which took advantage of spontaneous as well as carcinogen-induced immortalization of Hupki MEF, were carried out to establish a more systematic analysis of human *TP53* and mouse *p19Arf* alterations in Hupki MEF. Interestingly, Hupki MEF exposed to the smoking carcinogen B[a]P frequently showed mutations in the human *TP53* hotspot codons 157, 158 and 273, all of which have been linked to smoking-associated lung tumors.[24,25] In addition, homozygous deletion of murine *p19Arf* was also commonly found in the immortalized cells.[26]

Besides MEF, Syrian hamster dermal fibroblasts are another rodent cell type that has been used for its ability to immortalize following a clonal selection step, which results in the outgrowth of cell lines and functional selection for alterations in immortalization- and/or transformation-associated loci. In this setting, Cdkn2a/p16 locus deletion was found in cells treated with radiation or dimethylsulfate, mutations in the hamster *Tp53* and *Cdkn2a/p16* genes were associated with exposure to either B[a]P or MNU and treatment with the non-genotoxic carcinogen nickel resulted in Cdkn2a/p16 promoter methylation.[27]

In contrast to rodent cells, immortalization of human cells is an extremely rare event in the absence of viral oncogenes, but HMEC have been shown to immortalize solely due to carcinogen exposure.[15-17] HMEC immortalization is considered a key rate-limiting event in human carcinogenesis and, unlike finite lifespan HMEC,

overexpression of one to two known oncogenes can induce a malignant phenotype (anchorage-independent growth, growth factor independence, tumorigenicity) in immortalized cells.[28-30] HMEC immortalized by γ-irradiation showed *TP53* gene deletion, which resulted in loss of G1 arrest in response to radiation and tumor development in nude mice.[16] Another study used HMEC immortalization, initiated by N-ethyl-N-nitrosourea, as a surrogate for cell transformation, and allelotyping of 169 loci identified allele loss at 6q24-6q27 in both of the derived cell lines before any *TP53* mutations were observed. Interestingly, clonal allelic losses in this region were also found in morphologically normal terminal ductal lobular units microdissected from cases of in situ ductal carcinoma.[17]

Instead of using carcinogen-induced immortalization of primary cells as a functional readout for transformation of human cells, Damiani et al repeatedly exposed immortal HBEC to different carcinogens over a period of 12 weeks and assessed cell transformation using colony formation assays. Treatment with MNU and a combination of MNU and the reactive metabolite of B[a]P, BPDE, led to deletion of exon 2 of the *CDKN2A/p16* gene.[31]

Cell-based assays combining carcinogen exposure with a functional selection step have proven to be a valuable model system for recapitulating genetic alterations found in human tumors. To date, most work has focused on the study of single, well-known cancer-associated genes, and more recent, comprehensive genome-wide efforts of establishing mutation patterns in these models are discussed below.

Two recent studies in primary cells, one in MEF and one in HMEC, have taken advantage of the ability of these cells to clonally immortalize after carcinogen exposure (Figure 1A), a process that mimics the clonal development of tumors and permits mutation analysis by NGS at reasonable coverage and cost.[32,33] The study in MEF showed human tumor hotspot mutations in *Hras1* and *Kras* genes, in addition to recurrent alterations in multiple chromatin regulators implicated in cancer development and senescence regulation (*Dnmt1*, *Kmt2d*, *Arid1b*, *Arid2*, *Ep400*, *Kdm6b*).[32] Exome sequencing of HMEC immortalized by B[a]P treatment also revealed mutations with deleterious effects on protein function in several known and candidate cancer driver genes.[33] These mutations occurred in key regulators of cancer-related pathways, including chromatin and transcription (*MLL2*, *SETDB1*, *MED12*, *WHSC1L1*), cell cycle (*CDKN2A/p16*, *PML*), DNA repair (*FANCA*) and cell death (*CARD11*). In fact, most of these genes are known oncogenes or tumor suppressors.

The first comprehensive analysis of alterations in genes and pathways related to cancer development in these model systems was carried out using exome sequencing data from Hupki MEF that were immortalized following treatment with different carcinogens.[34] Almost 200 COSMIC CGC genes were found mutated, many of them recurrently, in a set of 25 cell lines. In total, 68 cancer and chromatin modifier genes were found recurrently mutated in the MEF lines, affecting pathways that regulate DNA damage response, DNA repair, cell cycle, cell death, transcription and chromatin structure, and developmental signaling. Functional impact of the mutations was further supported by the manifestation of several known cancer hotspot mutations among the identified alterations.

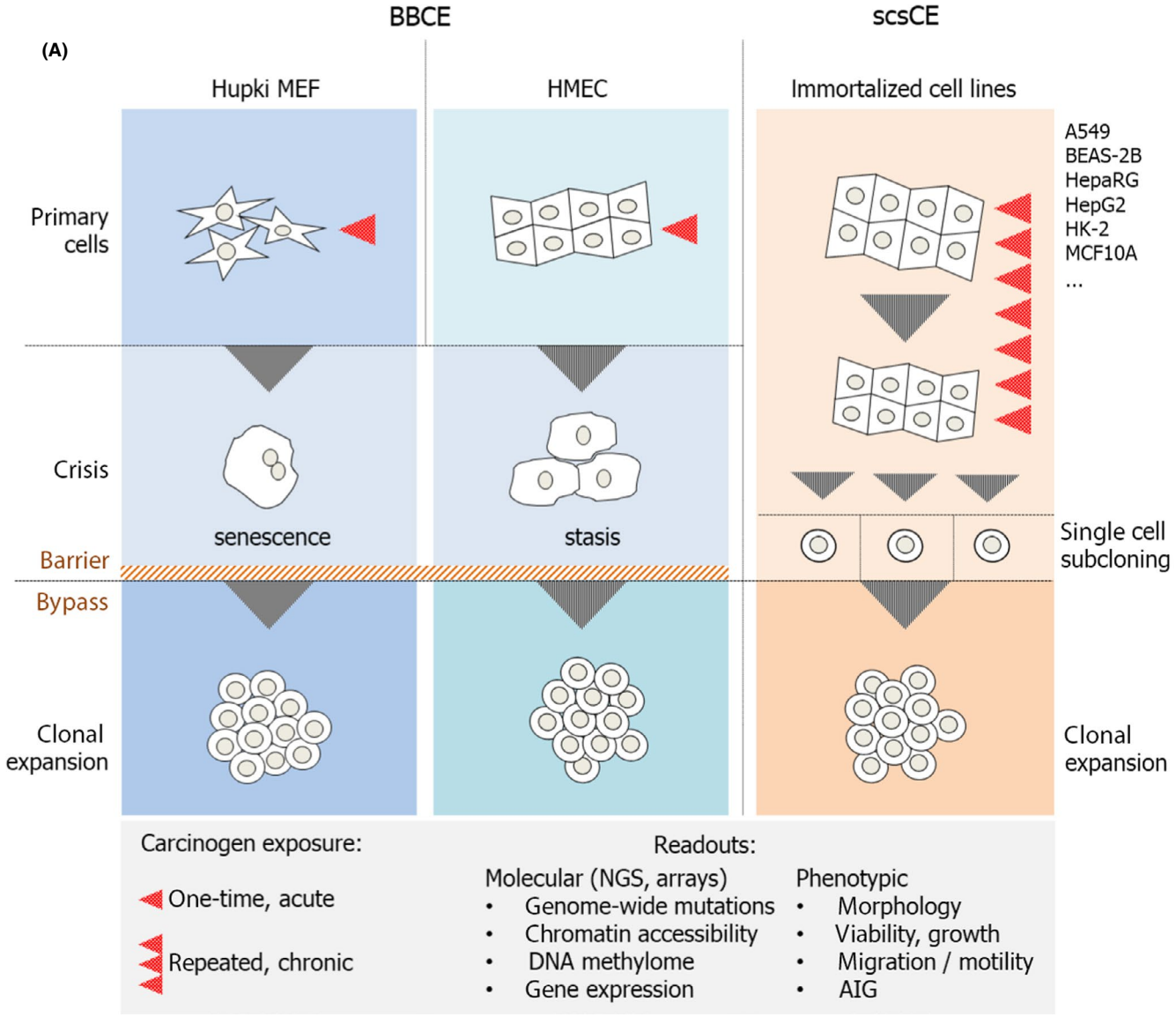
Applying a somewhat different approach, chronic carcinogen exposure of immortal human cell lines, rather than primary cells, combined with massively parallel sequencing of outgrown clones or single cell-derived clones can be used to characterize carcinogen mutational signatures (Figure 1A).[35-37] These studies generated a compendium of exposure-derived human epithelial cell clones with genome-wide mutation information. In contrast to cell clones derived using biological BBCE assays, mutations in the cell lines generated by circumventing this selection step through scsCE assays show limited overlap with cancer genes (Figure 1B). Interestingly, however, 30% of cancer gene mutations affect genes involved in chromatin regulation, hinting at an important role for epigenetic pathways in the context of carcinogen-induced stress and early stages of cell transformation (M. Korenjak & J. Zavadil, unpubl. data). Whereas the functional significance of mutations identified using such experimental models still needs to be verified, biological barrier-bypass models appear to yield cancer gene mutation profiles resembling more closely those found in human tumors (Figure 1B).

3 | CHROMATIN ALTERATIONS IN EXPERIMENTAL MODELS OF CELL TRANSFORMATION

Chromatin topography changes have emerged as a key mechanism for linking exogenous cues, such as carcinogen exposure, to DNA damage repair and gene expression changes, which can ultimately lead to cancer driver alterations and tumorigenesis. Such changes can be brought about, for example, by mutations in histone proteins and chromatin proteins involved in DNA repair, or by the loss of boundary function between transcriptionally active and repressed regions. Tumor sequencing studies have identified genes encoding chromatin-modifying factors as frequently mutated in cancer. In fact, the BAF chromatin remodeling complex has been shown to be one of the most commonly mutated human tumor suppressors.[38,39] In addition, DNA methylation changes are among the best-studied alterations associated with cancer development,[40] as cancer cells are characterized by global DNA hypomethylation, often accompanied by focal hypermethylation in the promoter region of tumor suppressor genes.

In experimental models, DNA methylation changes reminiscent of alterations found in cancer have been observed in senescent human as well as in immortalized mouse cells. An elegant study by Cruickshanks et al showed that global DNA methylation changes in senescent human lung fibroblasts recapitulate methylation changes reported in cancer (hypomethylation in late-replicating regions and lamina-associated domains, focal hypermethylation at CpG islands).[41] It was proposed that certain DNA methylation patterns characteristic for transformed cells are already present in premalignant senescent cells and can be propagated to cancer cells. Interestingly, among the genes with increased promoter methylation was the *CDKN2A/*

(A)



(B)

		Clones						
Model	**Exposure**	1	2	3	4	5	6	7
BBCE (mouse)	AA	24	16	14	12	9	7	16
	MNNG	41	25	36	48			
	Spont	8	13	3	10			
	AFB1	8	9	11				
	BaP	28	18	38				
	AID	13	13					
	UVC	7	35					
BBCE (human)	BaP	18	18					
	AA*	18	7					
scsCE (human)	AFB1	3	7	1	0	2	2	
	Cis	0	2	4	1	5	4	

FIGURE 1 A, Overview of the cell-based systems applicable to modeling of cancer driver events. Phenotypic progression for two barrier bypass-clonal expansion (BBCE) systems (primary human p53 knock-in [Hupki] mouse embryonic fibroblasts [MEF] and human mammary epithelial cells [HMEC]) are shown in the left and middle columns, respectively. A general schematic describing the single-cell subcloning-clonal expansion (scsCE) approach is depicted on the right. The immortalized cell lines used in the scsCE approach are derived from the lung (A549, BEAS-2B), liver (HepaRG and HepG2), kidney (HK-2) and breast (MCF10A). Other cell lines can be explored and developed for the scsCE approach. Treatment of the starting cell cultures with mutagenic carcinogens can be conducted as a one-time, acute exposure (single red triangles, top BBCE panels), or in a chronic, repeated method (typically for 8 weeks; multiple red triangles, top scsCE panel). The resulting clonal populations (bottom panels) are subject to high-throughput molecular and functional phenotypic analyses. AIG, anchorage-independent growth; NGS, next-generation sequencing. B, Cancer gene mutations in cell-based models of carcinogen-induced transformation. A comprehensive cancer gene list was curated from[5] and[8] Number shown for each exposure condition and clone indicates the number of mutations in known cancer genes. A statistically significant difference in the number of mutated cancer genes was observed between BBCE and scsCE assays ($P < .0001$, Mann-Whitney U test). AA, aristolochic acid; AFB1, aflatoxin B1; AID, activation-induced cytidine deaminase; BaP, benzo[*a*]pyrene; C; Cis, cisplatin; MNNG, N-methyl-N'-nitro-N-nitrosoguanidine; Spont, spontaneous; UVC, ultraviolet light type C. *Presumed barrier bypass based on clonal outgrowth following chronic AA exposure of immortal cell lines[35]

p16 tumor suppressor. A similar study in MEF identified a small number of common hypermethylated CpG in three independently immortalized MEF clones, several of which were associated with genes in the cancer-relevant Mek-Erk and Polycomb pathways.[42]

Similar to mutational changes, cancer-related chromatin alterations have been observed in cell-based models of carcinogen exposure and immortalization/transformation. Experiments in HMEC immortalized by B[a]P treatment showed stepwise changes in DNA methylation patterns when biological barriers (stasis, replicative senescence) are overcome.[43] Among the identified changes were hypermethylation of the *HOXA* and *PCDH* cancer gene clusters, reflecting observations in breast cancer, as well as of *CDKN2A/p16* and *PGR*, which are aberrantly methylated in atypical ductal hyperplasia.

Several studies from the Belinsky lab showed transformation-associated chromatin alterations following exposure of HBEC to MNU or a combination of MNU and BPDE. About one-third of the genes from a panel of 30 frequently methylated genes in primary lung tumors were methylated in exposed compared to untreated cells.[31] Moreover, the promoter regions of the tumor-suppressive microRNAs miR-200 and miR-205, as well as miR-196b, showed increased levels of the repressive histone marks H3K27me3 and H3K9me2 and of DNA methylation upon HBEC transformation.[44,45] These changes were associated with downregulation of miRNA expression. Interestingly, miR-196b promoter DNA methylation was also significantly increased in sputum of lung cancer cases compared to controls.[45]

Vaz et al transformed HBEC by chronic exposure (15 months) to cigarette smoke condensate,[46] which first resulted in an increase in repressive Polycomb histone marks at genes, followed by abnormal DNA methylation after 10 months of exposure. At 10 months, the cells underwent epithelial-to-mesenchymal transition, showed AIG and upregulated, oncogenic RAS/MAPK signaling. These cells could be transformed by single *KRAS* mutation, without other driver mutations, and formed lung tumors in mice.

It is evident that genetic and epigenetic mechanisms are interconnected at many different levels during tumor development. In addition to contributing to the silencing of critical tumor suppressor and DNA repair genes, 5mC modification of DNA favors hydrolytic deamination, which induces mutations due to deficient DNA mismatch repair, and it can also favor the occurrence of carcinogen-mediated DNA structures, such as adducts, which, in turn, cause defective DNA repair and mutations.[47,48] Moreover, histone modifications affect DNA repair by regulating chromatin accessibility as well as by creating binding platforms for repair proteins.[48] SBS tend to accumulate in heterochromatic regions in cancer genomes, a phenomenon at least in part attributed to reduced accessibility of the repair machinery.[49-51] Moreover, both DNA damage induced by controlled carcinogen exposure as well as tumor-associated genomic instability are determined by genome architecture.[52,53] Despite the progress in our understanding of these interactions, relatively little is known about the global relationship between specific mutational processes, genomic distribution of DNA damage and mutations, and their interplay with chromatin structure. Controlled experimental systems represent an intriguing model to discern the detailed mechanistic relationship between these processes.

4 | ADVANTAGES AND CAVEATS OF MODELING DRIVER EVENTS IN CELL-BASED SYSTEMS

Cancer-related mutational and chromatin changes have been observed in cell-based models of immortalization and transformation. This raises the possibility of taking advantage of these experimentally accessible systems to improve the functional understanding of alterations that drive the early events of cancer development.[54] With the emergence of CRISPR-Cas9 technology, the feasibility of correcting single point mutations or combinations in immortalized cell clones offers an intriguing opportunity for studying the contribution of mutations in putative cancer driver genes to the immortalized phenotype. Conversely, CRISPR-Cas9 screening approaches in primary cells, which do not spontaneously immortalize, may help to determine genes with critical roles during the immortalization process. In fact, shRNA knockdown of CDKN2A/p16 in primary HMEC facilitates the onset of the cell immortalization process,[55] and similar loss-of-function screens have been carried out in cell-based models to identify modifiers of cell migration and epithelial-to-mesenchymal transition.[56-59] In addition to the use of CRISPR, an ever-expanding

arsenal of chemical inhibitors that modify the activity of entire biological pathways or selectively target individual proteins provide yet another instrument to study the functional role of putative driver mutations. Using an inhibitor-based strategy, the cancer-specific functional relationship between BAF chromatin remodeling and PRC2 histone methyltransferase complexes was used in carcinogen-immortalized MEF. Cell viability and colony formation readouts showed a set of genes encoding subunits of the BAF chromatin remodeling complex that exhibited Ras-mediated dependence on PRC2 histone methyltransferase activity, a finding similar to what has been observed for other BAF subunits in cancer cells. Among the affected BAF complex subunits, *Smarcd2* and *Smarcc1* had not yet been identified as putative driver candidates by large-scale cancer genome-sequencing projects.[34] Hence, systematic analysis of genetic alterations identified in cell-based models of cell transformation can provide a valuable resource for future functional studies. Combining exposure to carcinogens that introduce characteristic mutation types with cell immortalization has the added advantage of using this information to help distinguish early events acquired before the barrier bypass from bystander events introduced later on.

Challenges remain in identifying/selecting the 'true' initiating events for subsequent elaborate functional studies. Use of current experimental models of cell immortalization for systematic cancer driver identification is limited by a number of factors. As a result of the moderate throughput of existing models and prohibitive cost for exome- or genome-wide mutation analysis, available datasets are relatively sparse. Therefore, systematic mining of the data is challenging. Nonetheless, analysis of a reasonably sized exome dataset, generated using MEF, showed a considerable number of recurrently mutated cancer genes, along with cancer-specific patterns of mutual exclusivity among the mutated genes from the same pathways.[34] In addition, a large number of immortalized cell lines have been generated in the past to study specific genes and pathways, and the drop in NGS cost may warrant re-evaluation of these archived cell lines. Despite ample evidence that supports the relevance of rodent cell models for studying the biology of human cancer, the use of non-human cells remains a caveat. As human primary cells cannot easily be immortalized spontaneously or by carcinogen exposure alone, availability of such models is unlikely to drastically increase in the future. Finally, some of the most commonly used experimental models, such as mouse embryonic and Syrian hamster dermal fibroblasts lack cell type relevance, as they do not share the epithelial origin of most cancers.

5 | OUTLOOK

Some of the currently pursued strategies to improve the experimental identification and validation of cancer driver alterations include refined experimental models as well as screening strategies. Clonal organoid cultures from multipotent cells offer an intriguing 3D culture model for studying the functional impact of putative drivers. They are amenable to NGS-based mutation analysis, and organoids derived from normal human adult stem cells showed mutations in several known cancer driver genes.[60] In analogy to 2D cell cultures, targeted alteration of candidate genes using the CRISPR-Cas9 system has been successfully implemented in organoids,[61,62] and targeted deletion of tumor suppressor genes alters cellular properties in vitro and heightens the transformation potential of organoids in vivo.[63] Over the last two to three years, a series of functional validation screens for VUS were developed based on the generation of barcoded expression clones for large numbers of variants.[64-67] Depending on the experimental setup, these screens can be carried out in vitro (immortalized cell lines) or in vivo (mice). They can be carried out in high throughput using a pooled setup or at a lower throughput for individual variants in order to avoid competition between mutations. Readouts for transformation capacity of mutants include expression profiling, cell viability assays, assessment of motility/migratory potential or in vivo tumor formation. Both organoid cultures and functional mutation screens in immortalized cell lines lack the unique selective barrier bypass associated with primary cultures, but they are nonetheless attractive experimental models for driver identification.

In the future, it will be important to combine experimental validation strategies in established barrier-bypass models with thorough genomic characterization of the derived cell lines and high-throughput functional validation screens, and also tap into the archives of toxicology programs that have generated tens of thousands of animal tumors, so far primarily for histopathological analyses. Ultimately, the use of diverse experimental approaches and models will only increase our ability to discern the functional impact of the multitude of putative driver genes and mutations identified by cancer genome sequencing, and to determine their contribution to tumor development.

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DISCLOSURE

The authors have no conflicts of interest, are alone responsible for the views expressed in this article and they do not necessarily represent the decisions, policy or views of the International Agency for Research on Cancer/World Health Organization.

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