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Phenotypic screening using large-scale genomic libraries to identify drug targets for the treatment of cancer (Review)

MITSUO SATO

Department of Pathophysiological Laboratory Sciences, Nagoya University Graduate School of Medicine, Nagoya, Aichi 461-8673, Japan

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Abstract. During malignant progression to overt cancer cells, normal cells accumulate multiple genetic and non-genetic changes, which result in the acquisition of various oncogenic properties, such as uncontrolled proliferation, drug resistance, invasiveness, anoikis-resistance, the ability to bypass oncogene-induced senescence and cancer stemness. To identify potential novel drug targets contributing to these malignant phenotypes, researchers have performed large-scale genomic screening using various *in vitro* and *in vivo* screening models and identified numerous promising cancer drug target genes. However, there are issues with these identified genes, such as low reproducibility between different datasets. In the present study, the recent advances in the functional screening for identification of cancer drug target genes are summarized, and current issues and future perspectives are discussed.

Contents

1. Introduction

Most human solid tumors develop through multi-step carcinogenesis (1,2). During this process, normal cells, presumed to be tissue-specific stem cells, accumulate multiple molecular changes advantageous to step-wise growth, finally transforming into overt cancer cells (3). Previous advances in genome-wide profiling technologies have revealed a number of molecular changes in malignant cells at the genetic, epigenetic, transcriptional and translational levels (4-6). Genetic alterations in proto-oncogenes that significantly contribute to malignant phenotypes are called driver oncogenes, such as activated forms of epidermal growth factor receptor (*EGFR*), *HER2/Neu* and *BRAF* (7-10). There are multiple drugs that successfully target these driver oncogenes. For example, *EGFR*-targeted drugs, such as gefitinib and erlotinib, exhibit anti-cancer activity against lung cancer with activating mutations in *EGFR* (11,12).

However, it is difficult to pharmacologically inhibit oncogenic signaling of some driver oncogenes. For example, the development of mutant *KRAS*-targeted drugs has proven problematic over the previous three decades (13). Although recently, treatment with AMG510, a novel inhibitor against *KRAS G12C*, resulted in a promising response rate in patients with lung cancer harboring this specific type of mutation, development of drugs targeting other types of *KRAS* mutations have not yet been successful (14-16). In addition, mutations in driver oncogenes in a number of types of human cancer have not been identified (17). In such cases, cancer results from non-oncogenes conferring various malignant phenotypes, occasionally in a context-dependent manner (18) and these genes may serve as novel therapeutic targets. For example, a study demonstrated that cancer cells depend on non-oncogene Heat shock factor 1 (HSF1), which is the master regulator of the heat shock response in eukaryotes, for their proliferation and survival than their non-transformed counterparts (19). To identify drug target genes for cancer cells harboring oncogenes which are difficult to pharmacologically inhibit, or do not have known oncogenes, it is vital to perform an unbiased, large-scale functional screening (20). Two important gene modulating technologies, RNA interference (RNAi) and clustered regularly interspaced short palindromic repeats-associated protein 9 (CRISPR-Cas9) have emerged as powerful tools for evaluating gene function (21). In addition, technologies in next generation sequencing have improved. The combination of these advanced technologies has allowed investigation of gene function at genome-wide levels in a high-throughput manner.

Thus, functional screening based on cancer-specific characteristics has been extensively conducted. In the

Correspondence to: Dr Mitsuo Sato, Department of Pathophysiological Laboratory Sciences, Nagoya University Graduate School of Medicine, 1-1-20 Daiko-Minami, Higashi-ku, Nagoya, Aichi 461-8673, Japan
E-mail: msato@met.nagoya-u.ac.jp

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majority of cases, functional screening is a four-step process: i) Inducing loss-of-function via RNA interference (RNAi) or CRISPR-Cas9 in cells; ii) evaluating the effects of the loss of the selected gene on phenotypes critical to cancer cells; iii) quantifying short hairpin RNAs (shRNAs) or single-guide RNAs (sgRNAs) via next-generation sequencing or microarray hybridization; and iv) data analysis (Fig. 1). Malignant phenotypes used for functional screening include uncontrolled promoted proliferation, drug resistance, invasiveness and the ability to bypass oncogene-induced senescence (OIS). In the present study, the recent advances in functional screening to identify cancer drug target genes have been summarized, and current issues and future perspectives have been discussed.

2. Types of functional screening according to phenotypes used

Dropout viability screening. Using genome-wide methodologies to identify target genes that substantially contribute to the uncontrolled proliferation of cancer cells is a straightforward approach to discovering cancer drug target genes for new drug development. This type of assay is called dropout viability screening. Two pioneering studies have conducted genome-wide dropout shRNA screening in various human cancer cell lines and identified genes essential for cancer cells (22,23). The Project Achilles study (launched in 2011) systemically identified genes essential for proliferation and/or survival in particular cancer cell types (genetic vulnerabilities) by performing an integrative analysis involving two steps: i) Conducting a pooled shRNA screen that targeted 11,194 genes in 102 (updated to 216 in the latest study) human cancer cell lines, including ovarian, colon, pancreatic, esophageal and non-small cell lung cancers; and ii) combining these results with information on alterations of cancer genome through using publicly available databases (24,25). By analyzing such diverse types of cancer, the study identified a number of lineage-specific essential genes. Another similar study used a pooled shRNA library comprised of 72 breast, pancreatic and ovarian cancer cell lines (26). In addition, after a CRISPR-Cas9-mediated gene-knockout technology became available in the experimental cell biology field (27), two studies demonstrated the feasibility of using lentiviral CRISPR-Cas9 libraries for functional screening, with certain advantages over RNAi libraries in efficacy and reliability (28,29). Via negative screening with RNAi or CRISPR-Cas9, these studies identified genes essential for proliferation in cancer cells, of which certain genes were lineage-specific.

One critical issue resulting from the nature of dropout viability screening is that such identified essential genes for cancer cells may also be essential for normal cells; for example, housekeeping genes involved in the ribosomal, proteasomal and spliceosomal pathways (26). Nevertheless, such essential genes may serve as promising therapeutic targets, as cancer cells highly depend on them for proliferation and/or survival compared with normal cells. One way to identify general essential genes that are likely to serve as cancer drug targets is to integrate results of genomic library screening with gene expression data and copy number changes between cancer and normal cells (20). This helps identify the genes that are associated with proliferation and/or survival in cancer cells (24). Using this approach, two housekeeping genes have been identified, proteasome 20S subunit alpha 6 (*PSMA6*; a proteasomal catalytic subunit) and eukaryotic translation initiation factor 2 subunit beta (*eIF2*β; a subunit of translation-initiation factor EIF2), as promising therapeutic targets for lung cancer (30,31).

Another way to identify essential genes that contribute to oncogenic phenotypes is to reveal the genes which cancer cells depend on in specific contexts; for example, with certain types of driver oncogenes (32). This situation is referred to as synthetic lethality and is described later. One study demonstrated that an essential gene *BUD31*, a component of the spliceosome is a potential therapeutic target specifically in MYC-driven cancers (33).

Synthetic lethality. A synthetic lethality refers to a phenomenon in which inhibition of one of two genes has no significant effects on cell viability but perturbation of both genes results in cell death (32). Synthetic lethality has attracted interest for the following reasons: i) If the synthetic lethality specifically occurs in cancer cells, treatments targeting genes involved in the synthetic lethality have a high therapeutic index; and ii) if the synthetic lethality involves driver oncogenes highly refractory to currently available treatment strategies, synthetic lethal genes may serve as good targets in types of cancer influenced by these oncogenes. A good example of such a gene is oncogenic *KRAS*, the most frequently mutated oncogene, although *KRAS*-targeted therapy is not used clinically (14). Using RNAi library screening, several studies have identified synthetic lethal genes in *KRAS*-mutated cancers, such as *STK33, TBK1, PLK1, SNAIL2, CDK1* and *GATA2* (34-39). However, these identified genes rarely overlapped between studies (40) and the identification of a synthetic lethal effect caused by *STK33* has not been reproduced (41,42). A recently conducted large-scale synthetic lethal RNAi screen, Project DRIVE, also failed to confirm significant synthetic interactions of mutant *KRAS* with these identified synthetic lethal genes (20). There are several possible reasons for such inconsistent results, including differences in methods of gene silencing (for example RNAi methodologies such as transient transfection of siRNAs or shRNA, and difference in types of library), and differences in types of cells used (for example variable dependencies on *KRAS* signaling). In particular, the latter seems to significantly influence screening results. Most studies of *KRAS* synthetic screens used cancer cell lines with or without mutant *KRAS* and/or isogenic cancer cell lines transfected with or without mutant *KRAS* (34-39). Cancer cell lines are highly variable in genetic changes (even those with the same driver oncogenes), which may result in inconsistent screening results (17,43).

Project DRIVE comprehensively assessed dependencies and synthetic lethal relationships using 398 cancer cell lines from different organs (20). To minimize false-positive rates, an average of 20 shRNAs per gene were used and, although synthetic lethal genes could not be confirmed for mutant *KRAS*, a number of novel findings regarding synthetic lethality which are translatable to developing novel therapeutics were identified. For example, reduced expression levels of an anti-apoptotic protein BCL2L1, and increased expression levels of pro-apoptotic protein BIM, were the

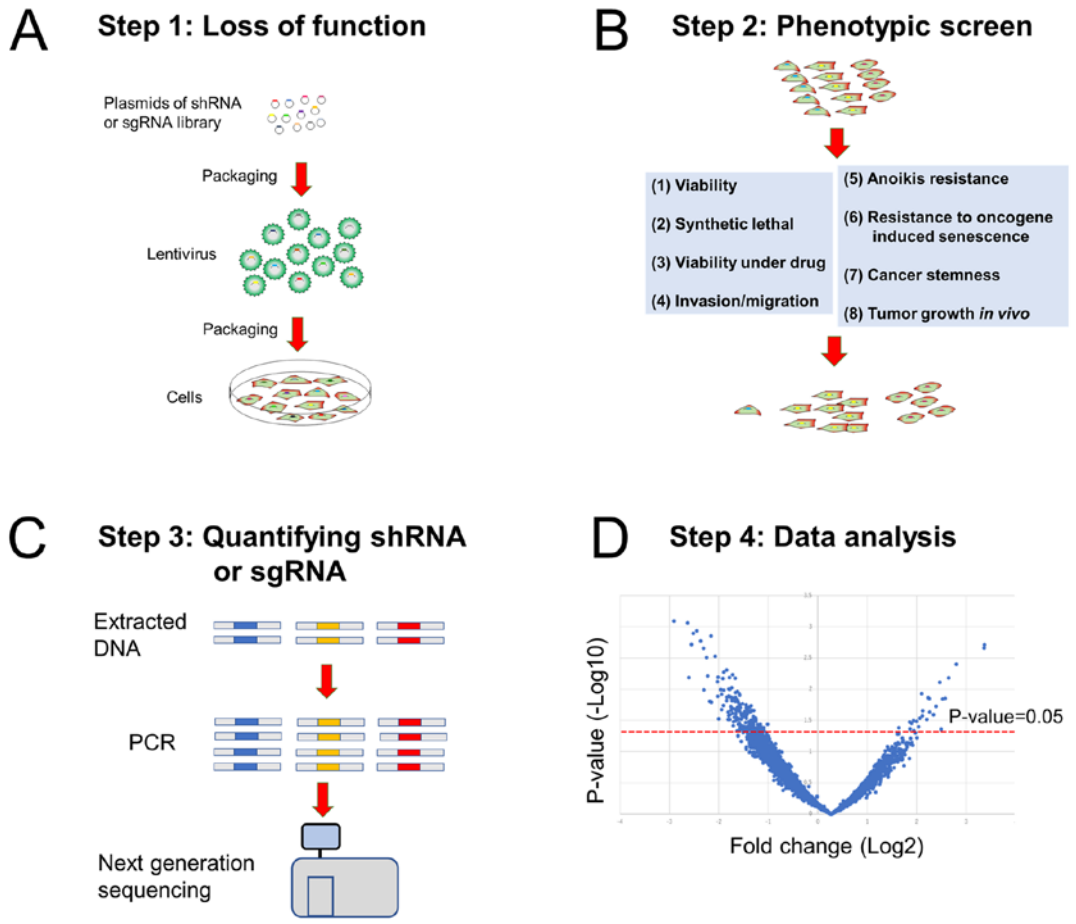

Figure 1. Flow diagram of the steps of phenotypic library screening with a genomic library for identifying cancer drug target genes. (A) Step 1: Loss of function, which is obtained by RNAi-mediated gene knockdown or Cas9-mediated gene knockout in cells. (B) Step 2: Phenotypic screen. Cells are subjected to various assays with different selection pressures including: 1, viability; 2, synthetic lethal; 3, viability under drug; 4, invasion/migration; 5, anoikis-resistance; 6, resistance to oncogene-induced senescence; 7, cancer stemness; and 8, tumor growth *in vivo*. (C) Step 3: Quantifying shRNA or sgRNA. DNA is extracted from harvested cells. Abundance of each shRNA or sgRNA is quantified using next-generation sequencing. (D) Step 4: Data analysis. Data are analyzed to generate ranked lists of promising cancer drug target genes. shRNA, short hairpin RNA; sgRNA, single-guide RNA.

strongest predictors of the growth-inhibiting effects following knockdown of anti-apoptotic protein myeloid cell leukemia sequence 1 (*MCL1*).

Recently, via genome-wide CRISPR-Cas9 screening, two independent groups identified WRN helicase as a synthetic lethal target in microsatellite unstable cancer types (44,45). Moreover, a small molecule inhibitor of WRN helicase (NSC617145) has been revealed to exhibit cytotoxic effects in cells derived from patients with Fanconi anemia, in a synthetic lethal manner (46).

Dropout viability screening under drug treatment. Drug resistance is a critical problem in chemotherapy (47). Cancer can be resistant to a number of types of drugs, such as cytotoxic, molecular-targeted drugs and immune checkpoint inhibitors (47-49). Therefore, researchers focus extensively on finding therapeutic approaches to overcoming the development of drug resistance.

Dropout viability screening in the presence of anti-cancer drugs is a powerful approach to identifying genes responsible for drug resistance and several potentially chemo-sensitizing targets have been reported (Table I). Using a genome-wide an arrayed RNAi library, Whitehurst *et al* (50) identified several genes influencing resistance to paclitaxel in a lung cancer cell line. Lin *et al* (51) identified *MCL1* as a potential drug target gene that sensitizes a small cell lung cancer cell line to ABT-737, an inhibitor of the antiapoptotic molecules Bcl-2, Bcl-X(L) and Bcl-w. After the development of pooled RNAi library technology, numerous investigators began using such libraries. For example, Prahallad *et al* (52) revealed genes responsible for resistance to a *BRAF* inhibitor PLX4032 (vemurafenib) in types of cancer harboring *BRAF* V600E mutations. It was revealed that *EGFR* activation, which is rapidly induced by vemurafenib treatment, induces resistance to vemurafenib treatment, suggesting that combination therapy of vemurafenib and an *EGFR* inhibitor may be beneficial. Previously, studies using CRISPR-Cas9 libraries were published. Most of these studies used the same type of genome-wide library, GeCKO CRISPR Library version 1 or 2, comprising of >120,000 sgRNAs targeting nearly the entire genome (53-56). For example, Sustic *et al* identified the endoplasmic reticulum to nucleus signaling 1 (*ERN1*)-*JNK-JUN* pathway as a potential target for improving the anti-cancer effects of MET inhibitors in *KRAS*-mutated colon cancer (56). *KRAS*-targeted therapy has not been successfully developed previously and, therefore, these findings are promising.

Table I. Studies identifying target genes for the treatment of cancer-associated drug resistance using large scale libraries.

Authors, year	Type of library	Size of library	Cancer type	Drug(s)	Identified genes or the pathways	(Refs.)
Bartz *et al*, 2006	Pooled shRNA	20,000 genes	Non-small cell lung, cervical and ovarian	Cisplatin	*BRCA1*, *BRCA2*, *TP53*	(97)
Whitehurst *et al*, 2007	Arrayed RNA oligos	21,127 genes	Non-small cell lung	Paclitaxel	*ACRBP*, *ATP6V0D2*, *FGD4*, *HS6ST2*, *PSMA6*, *TUBGCP2*	(50)
Lin *et al*, 2007	Arrayed RNA oligos	4,000 druggable genes	Small cell lung	ABT-737	*MCL1*	(51)
Lam *et al*, 2008	pooled shRNA	500 kinase genes	Diffuse large B-cell lymphoma	IKKβ inhibitors	*CARD11*	(98)
Xu *et al*, 2011	Arrayed RNA oligos	22,000 genes	Cervical	Cytotoxic nucleoside analog 2', 2'-diflurodeoxycytidine u	*SRSF3 SFPQ*	(99)
Guerreiro *et al*, 2011	Arrayed RNA oligos	719 kinase genes	Medulloblastoma	Cisplatin	*ATR*, *LYK5*, *MPP2 PIK3CG*, *PIK4CA*, *WNK4*	(100)
Liu-Sullivan *et al*, 2011	Arrayed RNA oligos	1,657 genes	Non-small cell lung	GSK461364A (PLK1inhibitor)	97 genes	(101)
Prahallad *et al*, 2012	Pooled shRNA	518 kinase and 17 kinase-related genes	Colorectal, prostate and thyroid	Vemurafenib	*EGFR*	(52)
Fredebohm *et al*, 2013	Pooled shRNA	1,000 genes	Pancreatic	Gemcitabine	*RAD17*	(102)
Milosevic *et al*, 2013	Pooled shRNA	779 kinase genes	Pancreatic	Erlotinib	*RPS6KA2*	(103)
Wetterskog *et al*, 2014	Arrayed RNAi	369 genes	ERBB2-ampfified breast	Lapatinib	*RAB34*, *TP53INP1*, *RAC1*, *ATP6C1V1*, *C11ORF73*, *MLLT6*, *NIBP (TRAPPC9)*, *NUFIP*, *PROCA1*, *RAB7L1*, *RAD21*, *SCRN2* and *SPOP*	(104)
MacKay *et al*, 2014	Arrayed RNA oligos	1,067 genes	Osteosarcoma	Cisplatin	*HOIP*	(105)
Maruyama *et al*, 2014	Pooled shRNA	10,000 shRNAs	Prostate	Bicalutamide	*RPL31*	(106)
Sudo *et al*, 2015	Pooled shRNA	16,000 genes	Non-small cell lung	gefitinib	*PRKCSH* and genes involved in the *CD27* signaling cascade	(107)
Prahallad *et al*, 2015	Pooled shRNA	298 phosphatases or phosphatase-related genes	Colorectal	Vemurafenib	*PTPN11*	(108)
Kobayashi *et al*, 2015	Pooled shRNA	~15,000 genes	Cervical, colorectal and non-small cell lung	2-deoxyglucose (2DG) (glycolytic inhibitor)	*COPB1*, *ARCN*	(109)
Yamaguchi *et al*, 2016	Pooled shRNA	2,924 genes	Head and neck squamous cell carcinomas	Rapamycin	Genes involved in the *ERK* pathway	(110)

Table I. Continued.

Authors, year	Type of library	Size of library	Cancer type	Drug(s)	Identified genes or the pathways	(Refs.)
Yamanoi *et al*, 2016	Pooled shRNA	~15,000 genes	Ovarian	Cisplatin	*ABHD2*	(111)
Kurata *et al*, 2016	Pooled CRISPR	19,050 genes	Acute myeloid leukemia	Ara-C	*DCK*, *SLC29A*	(53)
Hou *et al*, 2017	Pooled CRISPR	18,080 genes	Acute myeloid leukemia	FLT3 inhibitor AC220	*SPRY3*, *GSK3*	(54)
Sun *et al*, 2018	Pooled CRISPR	19,050 genes	hepatocellular carcinoma	Sorafenib	*SGOL1*	(55)
Sustic *et al*, 2018	Pooled CRISPR	65,383 sgRNAs	*KRAS*-mutant colon	MEK inhibitors	The *ERN1-JNK-JUN* pathway	(56)
Combes *et al*, 2019	Pooled CRISPR	518 kinase genes	Colorectal	Oxaliplatin	*ATR*	(112)

RNAi, RNA interference; CRISPR, clustered regularly interspaced short palindromic repeats; shRNA, short hairpin RNA; sgRNA, single-guide RNA.

Immune therapy using immune checkpoint inhibitors provides significant clinical benefit to patients with various types of cancer, including melanoma, lymphoma, and lung cancer (57). However, intrinsic or acquired resistance inevitably occurs, limiting the clinical benefits (49). Using genome-wide CRISPR-Cas9 or siRNA libraries, two studies identified *APLNR* (encoding the apelin receptor) and C-C motif chemokine receptor 9 as genes that may cause resistance to immune checkpoint inhibitors (58,59).

Invasion and migration. Metastasis is significantly associated with a poor patient prognosis, and patients with metastatic cancer exhibit poor survival outcomes (60). Metastasis comprises several sequential steps: i) Migration from a primary site; ii) intravasation; iii) passage by blood flow; iv) extravasation; v) and final settlement at distant sites. To complete this process, cancer cells must acquire the ability to invade and migrate and cancer cells exhibit these oncogenic properties. Previous studies demonstrated that epithelial-mesenchymal transition (EMT) significantly contributes to metastasis in cancer cells (61,62). EMT, and its reverse phenomenon MET, were initially identified during embryonic development, in which embryonic cells transform into terminally differentiated, specialized cells via several cycles of EMT and MET (61). A number of studies suggest a central role of EMT in metastasis (63-65). Previous studies have identified target genes for inhibiting migration and/or invasion ability of cancer cells through library screening. Pavan *et al* (66) developed a system combining RNAi library screening with a microscopy-based high-throughput quantitative analysis to identify a signaling pathway contributing to EMT in breast cancer. The group identified 59 genes whose inhibition suppressed transforming growth factor β-induced EMT in immortalized epithelial normal murine mammary gland cells. In addition, Pavan *et al* (66) focused on *MEK5* and *ERK5* belonging to the same signaling pathway and demonstrated the potential of targeting *MEK5* and *ERK5* as an anti-metastatic mechanism. Another study used migration ability as a phenotype for functional screening, identifying genes contributing to migration in glioblastoma, a highly invasive cancer (67). The authors performed a genome-wide RNAi screening in glioblastoma cells with a functional selection of cells able to migrate through Matrigel, identifying two genes [KH-type splicing regulatory protein *(KHSRP)* and host cell factor C1 (*HCFC1*)] as targets of invasion-suppressing therapeutics for glioblastoma.

Resistance to anoikis: Anchorage-independent growth. Upon detachment from the extracellular matrix or neighboring cells, normal epithelial cells undergo a type of apoptosis called anoikis (68). Anoikis prevents normal epithelial cells from colonizing at different organ sites, thereby maintaining the integrity of the body (68). Most cancer cells acquire resistance to anoikis, which is called anchorage-independent growth (AIG). The ability of AIG allows cancer cells to metastasize to different organs and is considered a hallmark of cancer cells (64). Several different molecular mechanisms underlying AIG have been identified, including the induction of intrinsic and extrinsic anti-apoptotic signaling, often triggered by changes in the expression patterns of integrin family members (68,69). In addition, previous studies have

demonstrated the role of EMT in AIG (68,70); however, the underlying molecular mechanisms of AIG are yet to be elucidated.

Eskiocak *et al* (71) used the immortalized untransformed colon epithelial cell line HCEC as a model system to evaluate the effects of shRNA-mediated knockdown of selected genes on AIG. The effects of the knockdown of 151 candidate cancer genes (*CAN*-genes), which have been identified as genes most likely to be drivers in breast and colorectal cancers, via a comprehensive statistical and bioinformatic analysis (72), were evaluated and revealed that *CAN*-genes are enriched in AIG suppressors. In addition, Simpson *et al* conducted a genome-wide shRNA screening to identify anoikis-resistant genes by culturing immortalized prostate and nasopharyngeal untransformed cell lines in a suspension condition, which identified α/β hydrolase domain containing 4 (*ABHD4*) as a promising target for inducing anoikis (73).

Resistance to oncogene-induced senescence (OIS). Activation of certain types of oncogenes, such as mutant *KRAS* and *BRAF,* causes normal cells to undergo senescence (74,75). This type of senescence is stress-induced and is termed OIS (74,75). OIS functions as a barrier to carcinogenesis initiated by normal cells, whereas senescence-associated secretory phenotype (*SASP*) is a carcinogenesis-promoting aspect of OIS (76). OIS was discovered by Serrano *et al* (77), who demonstrated that oncogenic *ras* induces premature senescence in experimental cell cultures. Subsequently, the occurrence of OIS in human disease was demonstrated in a developmental process of melanoma. Two studies revealed that OIS prevents benign melanocytic nevi, presumed to be the origin of melanoma, from transforming to overt melanoma (78,79). These studies demonstrated that proliferation of nevi cells is suppressed at very low levels despite harboring the highly oncogenic mutation *BRAFV600.* Studies have also revealed that *BRAFV600*-induced OIS is associated with p16INK4A upregulation (78,79); however, other unidentified changes may also be involved because of the complex mechanisms governing senescence (80). Therefore, several studies have attempted to the identify genes that may facilitate cells to bypass senescence induced by oncogenic *RAS* or *BRAF.* Vicent *et al* (81) performed RNAi screening to identify genes that facilitate bypassing ras-induced OIS in mouse models, reporting that Wt1 transcription factor (*Wt1)* is an OIS-bypassing gene using both *in vitro* and *in vivo* models. Vicent *et al* (81) also demonstrated *WT1* to be an independent prognostic factor in patients with *KRAS*-mutated lung cancer. Another study performed a near-genome-wide screening (~15,000 genes) to identify regulators of *BRAFV600*-induced senescence and identified *RASSF* as an OIS suppressor (82). Some screenings searching for OIS-bypassing genes used primary normal culture, and immortalized untransformed normal cell lines as model systems (82,83) because these cells are more prone to exhibit OIS, primarily due to their intact senescent machinery. However, the ability of cancer cells to undergo OIS may be impaired because of alterations in genes involved in senescence (74). Therefore, the applicability of the identified OIS-bypassing genes needs to be validated in multiple human cancer cell lines before exploring their usefulness as drug targets.

Cancer stemness. The cancer stem cell (CSC) theory hypothesizes that CSCs have the ability to self-renew and to differentiate into phenotypically diverse cancer cells (84). Although the CSC concept has not been demonstrated, accumulating evidence suggests that a number of types of cancer harbor CSCs (84,85). Notably, CSCs are hypothesized to be resistant to chemotherapy and irradiation (84). Therefore, the development of CSC-targeted therapeutics is attracting attention because of its potential to eradicate cancer cells. A functional library screening based on the sphere-forming ability of breast cancer cell lines identified *ATG4* as a promotor of the breast CSC-like phenotype (86). However, the usefulness of a sphere-formation assay for evaluating the self-renewal capacity is based on the assumption that the assay developed for normal neural stem cells can be accurately used for CSCs. Therefore, validation of genes identified as cancer stemness genes by other assays, such as a transplantation assays and lineage-tracing approaches, are required.

Genomic Instability. A phenotype of genomic instability facilitates diverse oncogenic properties because it causes numerous mutations resulting from the activation of oncogenic genes or inactivation of tumor suppressive genes (87). A previous study performed a genome-wide RNAi screen to identified the pathways and specific genes mediating genomic stability (88). A screen using elevation of γH2A.X variant histone (H2AX; a marker of double strand DNA damage) as an indicator for detecting DNA damage was conducted in HeLa cancer cells, identifying genes involved in DNA replication, checkpoint activation and DNA repair. The identified genes included *TIMELESS* and *TIPIN* encoding proteins that form a complex, leading to activation of the replication checkpoint. The identified genes may serve as promising drug targets to restore genomic stability in cancer cells (88).

Tumor growth in vivo. Tumor growth *in vivo* represents a more accurate screening method because it accounts for several aspects of real tumor growth, including 3-D condition, requirements for angiogenesis and the microenvironment (89).

One critical issue of *in vivo* pooled library screening is the difficulty in ensuring appropriate representation of the entire library in the initial population inoculated into model animals (89). There is a limitation to the maximum number of cells that can be inoculated, which limits the size and complexity of the library. Notably, the minimum cell numbers required for each shRNA differ significantly, depending on whether tumor suppressor genes or oncogenic genes are targeted for screening (89). In the case of oncogenic gene-targeted screening, low library representation tends to result in false-positive results, so instead of using genome-wide libraries, researchers used libraries focused on specific types of genes in order to identify oncogenic genes. For example, Singh *et al* (90) used an shRNA library of 150 genes associated with brain metastasis to conduct a library screening consisting of both *in vivo* (intracranial injection) and *in vitro* (tumor sphere-forming assay) assays to identify metastasis-promoting genes. The group successfully identified SPARC (osteonectin), cwcv and kazal like domains proteoglycan 1 (*SPOCK1)* and twist family bHLH transcription factor 2 (*TWIST2*) as

regulators of brain metastasis-initiating cells. In addition, most studies using *in vivo* genome-wide or near-genome-wide RNAi models discovered tumor-suppressive genes (91,92).

3. Conclusions and future perspectives

Shortly after RNAi technology for gene knockdown was developed in the laboratory, attempts to conduct large-scale functional screenings with RNAi were initiated (93). In addition, a gene knockout technique, CRISPR-Cas9 was also introduced for laboratory use (94). For >10 years, researchers have extensively conducted functional genomic screening to identify better targets and to develop new therapeutics for cancer. The present paper reviewed and summarized knowledge obtained by these studies, which has the potential to be used for drug development. Nevertheless, breakthroughs that can be immediately translated into clinical use are yet to be made. In particular, despite many reported studies, *KRAS* synthetic genes that have been reproducibly confirmed have not been successfully identified; therefore, development of *KRAS*-synthetic lethal drugs has not been successful. Project DRIVE suggested that no single synthetic lethal genes for *KRAS* exist. However, there may be certain strategies potentially enabling the identification of true *KRAS* synthetic genes; for example, one approach may be using more realistic modeling systems to evaluate malignant phenotypes. Such models may include a 3-D culture of cell lines and patient-derived xenografts (95,96), although such models are usually difficult to manage for large-scale screening. Due to the large heterogeneity in coexisting genomic alterations among *KRAS*-mutated tumors, studies using cancer cells may suffer from the presence of high background of noise during screening. Therefore, focus is needed on cancer cells which have higher similarities in harbored genetic alterations in addition to mutant *KRAS*.

An improvement in consistency of identified genes from a genome-wide screen has been revealed in CRISPR-Cas9 knockout compared with shRNA techniques (28). However, pharmacological inhibition of gene function with compounds is usually incomplete; thus, target genes identified through partial knockdown with RNAi represent improved targets. Therefore, results from CRISPR-Cas9 and RNAi screens need to be regarded as complementary.

In conclusion, advances in the technology of gene silencing and next generation sequencing have enabled researchers to conduct large-scale high-throughput phenotypic screenings, resulting in the identification of numerous potential novel drug targets for cancer. However, there are several issues, such as low reproducibility in the identified genes (40). Thus, substantial effort is required to adequately address these problems in order to identify novel cancer drug target genes.

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Availability of data and materials

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Author's contributions

MS designed the review, researched the literature and wrote the manuscript.

Ethics approval and consent to participate

Not applicable.

Patient consent for publication

Not applicable.

Competing interests

The author declares that he has no competing interests.

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