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RAPID COMMUNICATION

A controlled random gene perturbation method identifies *ARPC1B* gene as a key regulator of cancer metastasis

The discovery of clinically useful targets to treat specific pathological conditions can provide novel therapeutic approaches. To unbiasedly identify key genes related to tumor metastasis, we developed a random gene perturbation method using a *piggyBac* transposon system under the control of a doxycycline regulated promoter. Using this random gene perturbation method and utilizing a mouse model of metastatic pancreatic cancer, we identified genes dysregulated in metastasized cells from a random mutagenesis library after multiple rounds of *in vivo* selection. Analysis of these metastasized clones revealed the down-regulation of *ARPC1B* gene. Our further mechanistic studies revealed that *ARPC1B* gene and its closely related gene *ARPC1A* worked in a regulatory loop to control tumor metastasis. These findings validate that *piggyBac* transposon mediated random gene perturbation is a powerful tool to investigate the functional relevance of novel genes and the *ARPC1A/B* axis is a potential key regulator of tumor metastasis.

Despite significant advancement in our understanding and development of novel therapeutics, mortality due to cancers, especially those metastasize to the secondary organs, remains high.[1] Although both forward and reverse genetics have identified many genes that are associated with the metastatic process,[2] our understanding of cellular and molecular pathways that contribute to or limit the metastatic process is far from complete.

The development of novel approaches that insert random gene mutations has provided an unbiased approach to discovering specific gene functions relevant to specific pathologic conditions.[3] Here we used a novel random gene perturbation method to generate a library of cells with insertion mutations, in combination with a mouse model of cancer metastasis to identify key genes dysregulated in the metastasized tumor compared to the parent cell population.

To develop a functional genetic screen, we combined *piggyBac* transposon mutagenesis,[4] Tet-inducible expression system and antisense RNA technology. The *piggyBac* transposon-based gene search vector (GSV) contains a tetracycline-regulated element (TRE) regulated promoter, a G418 selection marker and a plasmid replication origin, all of which are flanked by the PB terminal DNA repeats (Fig. S1A). With the help of transposase, the GSV can integrate into the cellular genome. The gain-of-function or loss-of-function mutagenesis, initiated by the sense or antisense RNA, was determined by the integration orientation of GSV and regulated by tTA. In the absence of tetracycline, the tetracycline transactivator (tTA) binds to TRE and initiates the sense or antisense transcription, then enhances or blocks the target gene expression (Fig. S1B). The addition of doxycycline will reverse the production of either sense or antisense RNA by detaching tTA from TRE, thus reverse the target gene expression and phenotypes. To establish a Tet-off AsPC-1 human pancreatic cell line, we used luciferase reporter assay as the screening tool. Clone 2D2 possessed high transactivator activity, which was effectively suppressed by doxycycline (Fig. S1C). To generate a gene mutagenesis library, the amount of GSV and transposase were tittered (Fig. S1D, E) and the cells were selected in medium containing G418 for 2 weeks. In the absence of transposase, very few G418 resistant colonies were generated, potentially resulting from the random insertions of GSV in the genome. In contrast, co-transfection of GSV with transposase generated thousands of G418 resistant colonies (Fig. S1F). These mutated clones were injected into the BALB/c nude mice by intravenous (*i.v.*) injection and the cells that metastasized to the lung were isolated *in vivo* by primary cell culture using G418 selection (Fig. 1A). Multiple rounds of *in vivo* selection were

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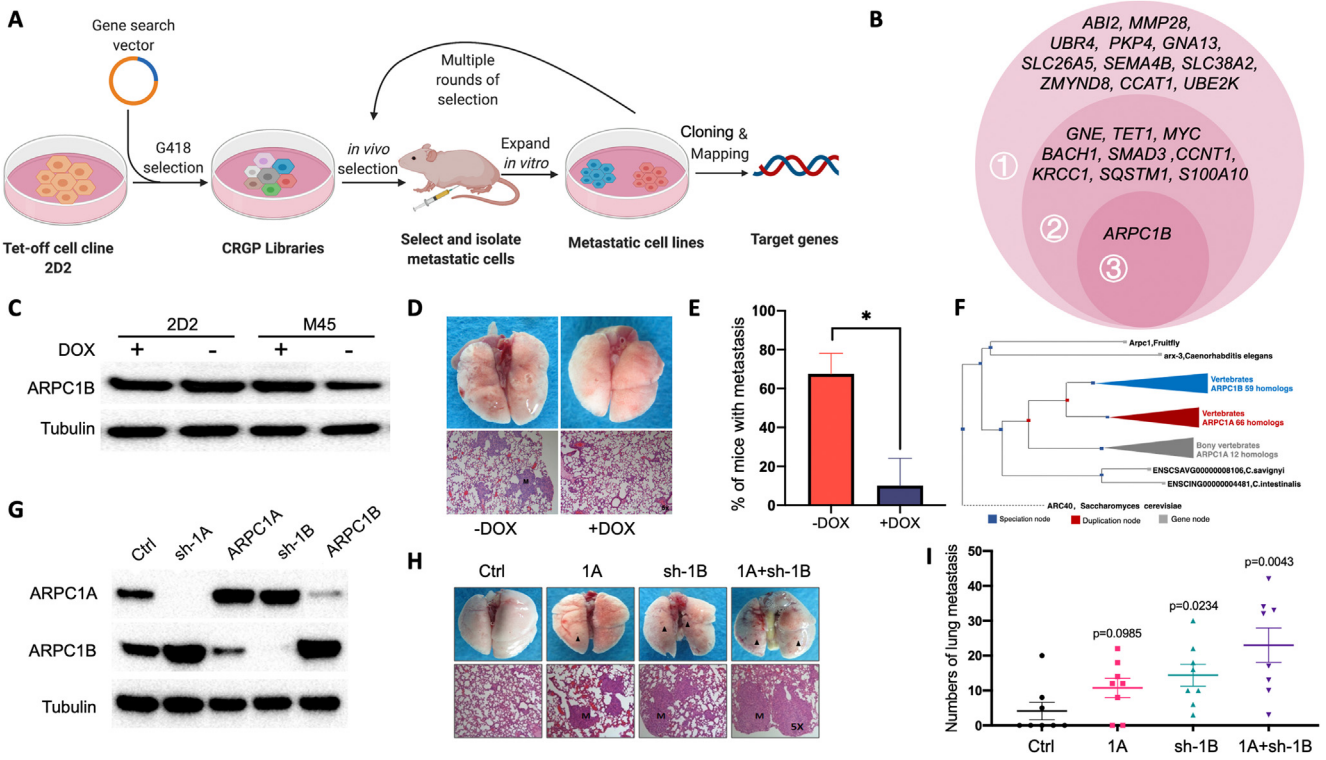

Figure 1 Identification of *ARPC1B* gene as a key regulator in cancer metastasis. **(A)** Flow chart of the experimental approach. **(B)** Genes identified from lung metastasized G418 resistant clones by Splinkerette PCR in three rounds of screening. **(C)** Decreased levels of *ARPC1B* in clone M45 and its negative regulation by doxycycline (DOX). This regulation was absent in parent clone 2D2. **(D, E)** Lung metastasis of clone M45 was inhibited by doxycycline. Representative gross lung pathology and histological staining are shown (D). The quantification of mice with metastatic lung tumor in the presence or absence of doxycycline is shown (E). **(F)** The gene tree of *ARPC1B* was constructed using ENSEMBLE, genetic evolutionary tree analysis identifying gene bifurcation resulted in *ARPC1A* and *ARPC1B* in mammals. **(G)** Silencing of *ARPC1B* increased the expression of *ARPC1A* and *vice versa* in pancreatic cancer cell line ASPC-1. **(H, I)** *In vivo* experiments confirmed that the overexpression of *ARPC1A* and the low expression of *ARPC1B* could significantly increase the number of pulmonary metastases (H). The quantification of mice with metastatic lung tumor in ORF-*ARPC1A* group, sh-*ARPC1B* group and ORF-*ARPC1A* + sh-*ARPC1B* (I).

performed, and the metastasized clones were obtained for subsequent analysis.

In the first round, 28 metastasized clones were identified, the genomic DNA was extracted, and Splinkerette PCR was performed to clone the candidate genes, in total 21 candidate genes were obtained. In the second round, 10 candidate genes were identified and in the third round, only actin-related protein 2/3 complex subunit 1B (*ARPC1B*) gene was obtained (Fig. 1B). We then investigated the location of the candidate gene to identify the insertion site of GSV. GSV in the last round of clones was found to be integrated into the intron of *ARPC1B* gene and initiated the transcription to produce antisense RNA, thus blcoking *ARPC1B* expression. The Splinkerette PCR products in the process of cloning *ARPC1B* were found to be located in the same location (Fig. S2A—D). To confirm the integration site of GSV, the genomic PCR was performed (Fig. S2E). Interestingly, many of the other clones found in this screen (*SMAD3*, *S100A*, *MYC*, *SQSTM*, *BACH1*, *etc.*) have been reported to be related to cancer metastasis, supporting the validity of our screening method. Insertion sites of GSV in these genes are indicated in Table S1.

To validate the differential expression of *ARPC1B* gene in metastasized tumor cells, we measured the protein levels of ARPC1B and its regulation by doxycycline in metastasized clone M45 and parent cell line 2D2. We found that decreased expression of *ARPC1B* in clone M45 was reversed by doxycycline treatment (Fig. 1C). To validate the casual relation of *ARPC1B* gene to the metastatic phenotype, the nude mice were injected with clone M45 *i.v.* and then divided into two groups with or without oral doxycycline administration. We observed that doxycycline treatment significantly decreased the lung metastasis of clone M45 (Fig. 1D, E). Moreover, *in vitro* cell migration assay demonstrated that doxycycline treatment decreased the migration ability of clone M45 (Fig. S2F, G).

Actin-related protein 2/3 complex subunit 1A and 1B are alternative subunits of ARP2/3 complex.[5] Blast analysis showed that human *ARPC1B* has 68% homology with human *SOP2L* (*ARPC1A*). In *Saccharomyces cerevisiae*, *Caenorhabditis elegans*, *Drosophila melanogaster*, and other lower organisms which have ARP2/3 complex homolog, *ARPC1A* and *ARPC1B* orthologous genes exist as a single gene (Fig. 1F).

Next, we investigated potential interaction between *ARPC1B* and *ARPC1A* and how this interaction affects the metastasis phenotype in cancer cells. We constructed overexpression and knockdown plasmids of *ARPC1A* and *ARPC1B* using the lentivirus packaging system LV-EF1α-ORF and PLL3.7, respectively. The results showed that *ARPC1B* overexpression downregulated the level of *ARPC1A*, and *ARPC1B* down-regulation led to increased expression of *ARPC1A* in ASPC-1 cells, and *vice versa*, indicating a regulatory loop between *ARPC1A* and *ARPC1B* (Fig. 1G). To understand the contribution of *ARPC1A* in cancer metastasis, we constructed three groups of cells including ORF-*ARPC1A* group, sh-*ARPC1B* group and ORF-*ARPC1A* + sh-*ARPC1B* group along with the control group using ASPC-1 cell line (Fig. S2H). The cells were then injected into the nude mice. Histological analysis demonstrated that ARPC1A overexpression promoted lung metastasis, and overexpression of *ARPC1A* along with knockdown of *ARPC1B* further increased the disease severity. In addition, either overexpression of *ARPC1A* or knockdown of *ARPC1B* aggravated lung metastasis, which was further increased in ORF-*ARPC1A* + sh-*ARPC1B* group (Fig. 1H, I). To further explore the mechanism of *ARPC1A* contributing to metastasis, we used a Transwell system to detect cell migration ability across the Transwell chamber. The results showed that both overexpression of *ARPC1A* and knockdown of *ARPC1B* significantly enhanced the invasiveness of cells *in vitro* as manifested by increased migration ability (Fig. S2I, J) indicating potential mechanisms by which *ARPC1A* promotes tumor metastasis.

Finally, to determine the clinical relevance of *ARPC1A* gene expression level for prognosis of cancer, we extracted expression data of 177 pancreatic adenocarcinoma (PAAD) patients from PanCancer Atlas in The Cancer Genome Atlas (TCGA) consortium and plotted Kaplan-Meier curves with a web-based survival analysis tool (http://kmplot.com/analysis/index.php?p=background). Our data showed that high expression of *ARPC1A* was inversely correlated with the survival time of PAAD patients validating the role of ARPC1A in the development and progression of pancreatic cancer (Fig. S2K, L).

Together, this study not only establishes and validates the use of the *piggyBac* system to generate random mutagenesis to identify novel therapeutic targets in specific pathogenic conditions, but also provides a conceptual framework for identifying and functionally dissecting the master regulators in human diseases including cancer.

Conflict of interests

The authors declare that there is no conflict of interests.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.gendis.2022.06.006.

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