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RAPID COMMUNICATION

Lung adeno-squamous carcinoma modeling using oncogenic KRAS activation in human embryonic stem cell-derived alveolar organoids

Mutant KRAS is a common driver in lung cancer. Nevertheless, the occurrence of human lung cancer and its subsequent development after activation of oncogenic KRAS in epithelial cells remain poorly understood. Organoids are embedded in three-dimensional Matrigel in which tissue-derived human adult stem cells can efficiently grow for a long time, maintaining genetic and phenotypic stability.[1] But until now, the use of organoids to simulate human non-small cell lung cancer has not been reported.

To generate $KRAS^{G12D}$ mutated hESCs, we used CRISPR/Cas9-based genome editing and Cre-LoxP system to induce somatic G12D heterozygous mutation in the *KRAS* gene, with normal RNA splicing, and maintaining normal pluripotency and karyotyping (Fig. S1). By using the platform of human lung alveolar organoids which we have reported previously[2] (Fig. 1A), $KRAS^{G12D}$ mutated organoids appeared with distinct morphology from wild-type (WT) after exchange maturation medium (Fig. 1B). About 95% WT lung alveolar organoids were cystic, consisting of a monolayer of polarized epithelial cells surrounding a large central lumen. By contrast, around 87% $KRAS^{G12D}$ mutated organoids appeared dark, with convoluted invaginations, showed different nuclei shapes and sizes (Fig.1C; Fig. S2A).

Next, we found that about 43% Edu $^{+}$ cells in the organoids with $KRAS^{G12D}$ mutation, while only about 6% in WT (Fig. 1D). Through analysis of cell count during organoids culture, we found the proliferation rate of organoid cells and alveolar organoid size with *KRAS* mutation were significantly increased (Fig. S2B–D). $KRAS^{G12D}$ organoids highly expressed DNA replication related genes (Fig. S2E). Through digesting the organoids into single cells, the sizes of the grown-up *KRAS* mutated organoids were larger than WT after 10 days (Fig. S2F, G). Of note, the grown-up *KRAS* mutated organoids showed heterogeneous shapes, while WT were even, which might reflect the heterogeneity feature of lung cancer. At the same time, we found that the *KRAS* mutant organoids contained a mass of apoptotic cells in lumen (Fig. S2H), which was also consistent with HE staining (Fig. 1C).

To identify global transcriptomic changes occurring upon KRAS activation, we performed RNA sequencing of $KRAS^{G12D}$ and the parental organoids at different differentiation times (Fig. S3A, B). Surprisingly, we found a downregulation of specific markers of differentiation and maturation (such as *SOX2* and *NKX2.1*) and of AT2 functions (such as *SFTPB* and *SFTPC*) (Fig. 1E; Fig. S3C, D). Instead, some progenitor and developmental markers (such as *ETV4*, *ETV5* and *SOX9*) and some genes that stimulated the proliferation of NSCLC cells (such as *FOXQ1* and *ID1*) were upregulated (Fig. S3C–E), suggesting $KRAS^{G12D}$ mutated cells might transition to the developmental-like state. As we all known, the cytoplasmic localization of SOX9 was also positively correlated with the malignant degree of the tumor. Interestingly, we found SOX9 expression was upregulated and located in cytoplasm upon $KRAS^{G12D}$ (Fig. 1E).

Among gene ontology (GO) analysis (Fig. S4A), we found that the differential genes were concentrated in extracellular matrix, as well as cell differentiation, development, and proliferation. Western blot showed the expression of phosphorylated ERK1/2 was increased, indicating constitutive activation of KRAS downstream signaling pathway (Fig. 1F). We also found the corresponsive genes of Wnt signaling pathway were all upregulated (Fig. S4B), which also in line with the response of early tumor occurrence. Interestingly, we found that the expression of many genes involved in extracellular

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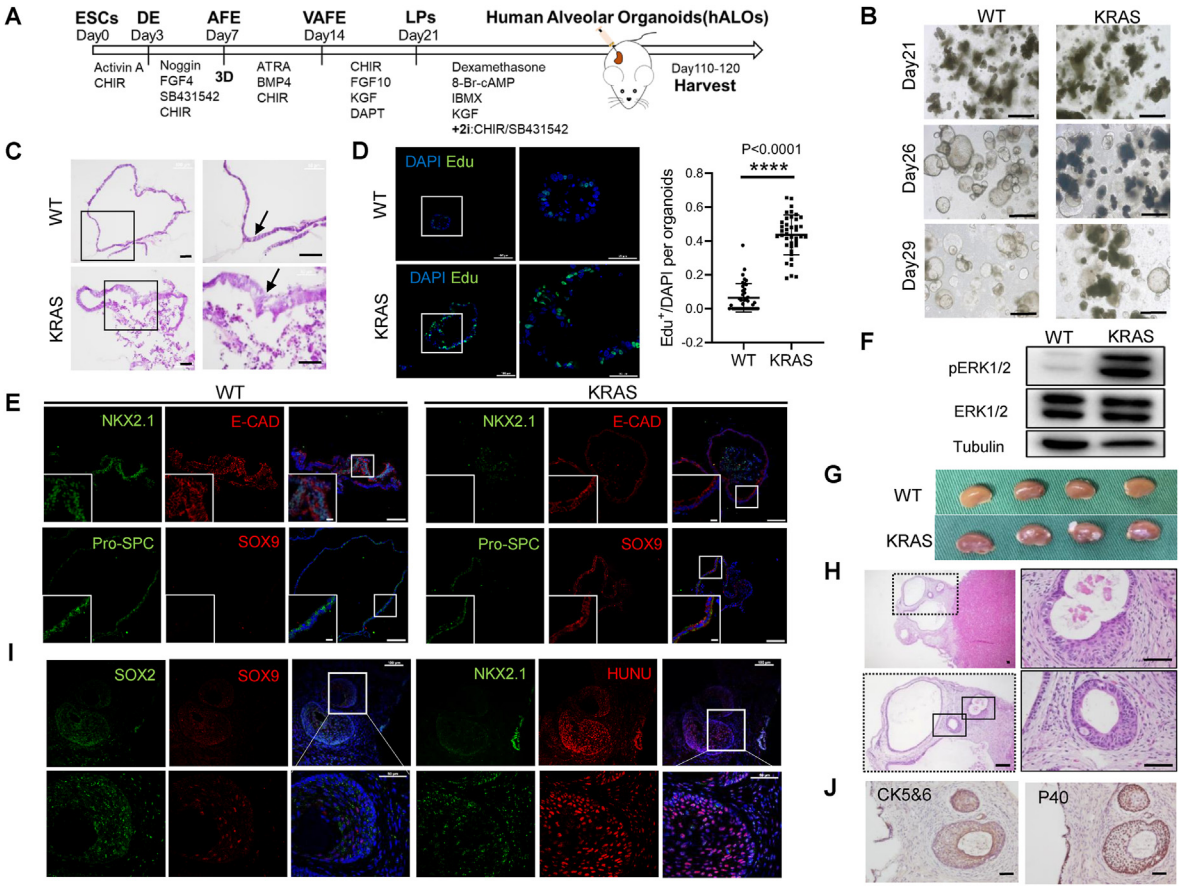

Figure 1 *KRASG12D* played an important role in the initiation stage of lung adeno-squamous tumorigenesis. **(A)** Schematic of protocol and stages from hESCs to human alveolar organoids (hALOs). **(B)** Representative images at the indicated differentiation stages of WT and KRAS mutated hESCs. Scale bar: 500 μm. **(C)** HE staining of WT and KRAS mutant organoids. Scale bar: 50 μm. **(D)** Edu staining of WT and KRAS mutated alveolar organoids cultured *in vitro*. Scale bar: 100 μm. The right plot showed the percentage of Edu+ cells per organoid. At least 40 organoids were counted. **(E)** Immunofluorescence staining of alveolar organoids on Day30. Scale bar: 100 μm. E-CAD (E-cadherin) is a marker of epithelial cells. Pro-SPC is a marker of alveolar type 2 cells. **(F)** Phosphorylated ERK1/2 and total ERK1/2 expression level of WT and KRAS mutated alveolar organoids were detected by Western Blot. **(G)** The kidneys of mice were harvested and the grafts were shown. No graft growth was observed in the kidneys transplanted with WT organoids and small grafts were observed in the kidneys transplanted with *KRASG12D* organoids. **(H)** HE staining of *KRASG12D* organoid grafts. Scale bar: 100 μm. **(I)** IF staining for lung progenitor cell markers (SOX2, SOX9, NKX2.1) of *KRASG12D* organoid grafts. Scale bar: top 100 μm, bottom 50 μm. **(J)** IHC staining of lung cancer-related markers of *KRASG12D* organoid grafts. Scale bar: 50 μm.

matrix, which were recognized as a regulator of cell proliferation and metastasis, was significantly altered with *KRAS* mutation (Fig. S4C, D).

The reprogramming of cellular metabolism is a hallmark of cancer, to support the energetic needs of cell uncontrolled proliferation. Glutamate metabolism, lipid metabolism, carbohydrate metabolism and amino acid metabolism were the main dysregulation process in our lung organoids upon $KRAS^{G12D}$ (Fig. S5).[3] Upregulation of fucosyltransferase (FUT) family expression has been associated with the induction of metabolic changes, and then FUT9 has been remained as a metabolic driver of advanced colon cancer.[4] We found the expression of *FUT3/6* and *FUT9* were up-regulated upon KRAS activation (Fig. S5C). Through the glutathione metabolic analysis (Fig. S5D, E), we found that chloride intracellular channel protein (CLIC) family and glutathione S-transferase (GST) family genes were down-regulated. Lipid metabolism analysis showed up-regulated expression of sphingosine kinase 1 (SPHK1) and sphingosine-1-phosphate phosphatase 2 (SGPP2), especially at late stage (Fig. S5F, G), indicating active sphingomyelin uptake and activation process.

From the results of differentiation as mentioned above, the organoids could not evolve the malignant degree of the tumor. Therefore, organoids transplanted under the kidney capsule of NSG mice after 110—120 days, we found that grafts grew obviously upon KRAS mutation, with vacuolar and glandular structure, irregular cell arrangement and nuclear atypia, reflecting certain characteristics of cellular carcinogenesis (Fig. 1G, H). According to the clinical classification, NKX2.1 (TTF1) was positive, while SOX2 and SOX9

were weakly positive, indicating the characteristics of lung adenocarcinoma (Fig. 1I). CK5&6 and P40 were also positive (Fig. 1J), indicating the expression of markers of lung squamous carcinoma. At the same time, as shown in Figure S6, some areas within the grafts showed HUNU and lung epithelial markers, as well as high expression of lung adeno-squamous carcinoma markers.

As we all know, the development of a tumor needs mesenchymal cells to provide microenvironment. Therefore, we used another differentiation platform reported previously (Fig. S7A)[5] to test whether *KRAS* mutant lung organoids could develop into a higher degree within the heterogenetic cell niche. Bright field showed that organoids did not form vacuolar structures, but spherical structures with many mesenchymal cells (Fig. S7B). qPCR determined the differentiation of organoids (Fig. S7C), but the efficiency was much lower. After transplanting organoids under the kidney capsule for about 120 days, significant grafts were harvested (Fig. S8A), with irregular alveolar and squamous epithelioid structures, special atypia nuclei and highly expressed lung adeno-squamous carcinoma markers (Fig. S8B—E). At the same time, we tried orthotopic transplantation of organoids at different differentiation time points. Unfortunately, we only observed scattered cells, not clonal populations in lung (data not shown).

In this study, using human lung organoids, we found that compared with WT, the proliferation and apoptosis of $KRAS^{G12D}$ lung epithelial cells were significantly active, the frequency of cell renewal was accelerated, and the cells were not tightly arranged, which were in line with the early stage of the tumor. We firstly found cancer-associated pathways were activated and metabolically disordered in organoids. Surprisingly, we found the localization of SOX9 was transferred from the nucleus to cytoplasm, which were consistent with the feature of NSCLC. However, after transplantation under the kidney capsule, the grafts mimicked the poorly differentiated lung adeno-squamous carcinomas with the heterotypic nucleus. And orthotopic lung transplantation did not show cloned-like tumor. So, we guess $KRAS^{G12D}$ mutation is not enough to malignant lung cancer. Our organoids provide an alternative method to intervene in the accelerated progression of lung cancer. Using the organoid platform, instead of patient-derived cancer organoids, we can study the initial event of lung cancer and how genetic damage induces the initiation of cancer. We suggest that this organoid system can be used for *in vitro* operation and subsequent transplantation, for the study of potential therapeutic targets of lung cancer.

Author contributions

Z.R., Y.L., and J.F. designed the experiments, analyzed the data, and wrote the manuscript. J.F., L.L., S.Z., and X.Z. performed experiments. Z.T. performed the bioinformatics analysis. J.W. and Y.Y. helped to analyze H&E and IHC data. Z.R. and Y.L. provided financial support.

Conflict of interests

The authors declare no competing 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.2022.03.015.

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