A Human Pluripotent Stem Cell-based Model for Lung Carcinoid

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Introduction

Pulmonary carcinoids including typical(TC) and atypical carcinoids(AC), are rare neuroendocrine tumors(NETs) that constitute around 2% of all primary pulmonary malignancies. Their incidence has notable increased compared to NETs in other sites in recent decades, however, there is no major advance in treatment and prognosis for these cancers. This reflects our limited understanding of the biology of lung carcinoid; and current research of pulmonary carcinoid has been hindered by the scarcity of primary tumors and established cell lines, due to its low prevalence.

Based on the neuroendocrine characteristics, pulmonary neuroendocrine cells (PNECs) are the putative cell of origin of lung carcinoids, especially vulnerable to transformation by genetic mutations implicated in the malignancies. However, it is difficult to directly study the carcinogenesis of PNEC, due to several obstacles: PNECs are rare airway epithelial cells, and there is no effective *in vitro* culture system that enables growth and spread of primary PNECs. Genetically engineered animal models have many advantages; however, in many instances certain hypotheses cannot be tested due to species difference. As a result, there is scant knowledge except at the most descriptive level about the cellular or genetic mechanism of lung carcinoids. **Therefore, new models that recapitulate the clinical features of lung carcinoid could be critically important for advancing our knowledge of the disease and developing effective therapies.**

In our previously published research, we have established, to our knowledge, the first method to produce high proportions of PNEC by differentiation of human pluripotent stem cells (hPSCs). We further demonstrated the feasibility of the cells in modeling neuroendocrine (NE) tumor, by alterations of *RB*, *P53* and *C-MYC*, the driver genes in small cell lung cancer (SCLC). The hPSC-derived PNECs were able to undergo oncogenic transformation *in vitro*, and form SCLC-like tumors when implanted in immunodeficient mice. **The availability of hPSC-derived PNECs paves a new avenue for modeling and studying lung NE tumors including lung carcinoids.**

Objective

This project aims to create novel disease models as needed research resources for advancing understanding of the biology and developing new treatments for pulmonary carcinoids. To approach these problems, we propose here to exploit our novel methods for generating, isolating, and characterizing PNECs and their committed progenitors; for altering the genetic factors or signaling pathways recurrent in lung carcinoids; and for appraising the manifestations of neoplasia in culture and in animals. We will further define the similarities between the genetic and physiological features of the lung carcinoids from hPSCs and the ones in patient samples. Through these studies, we expect to establish innovative pulmonary carcinoid models with human cell origin that recapitulates the major clinical features of these tumors. Once succeed, this feasible and trackable model system will enable studying a broad range of questions about biology of pulmonary carcinoid, and serving as an innovative platform for therapeutic studies.

Background

Limited Models or Resources for Studying Pulmonary Carcinoids

Pulmonary carcinoids are rare neuroendocrine tumors of the lung. Resection is often an effective treatment for early stage diseases, however, no standardized and effective adjuvant therapy has been established for patients with advanced unresectable diseases. Recently developed targeted therapy such as mTOR inhibitors, has achieved breakthroughs for pulmonary carcinoids. However, the progression of therapy is still limited, indicating an in-depth understanding of disease mechanism is imperative. Primary tissues, disease models and cell line resources to study the biology of pulmonary carcinoid are currently scarce, and the existing cell models (ATCC cell lines) cannot fully recapitulate the disease features. Therefore, new models and tools are urgently needed for studying the biology and novel treatment approaches for these rare tumors.

Human Pluripotent Stem Cell-Based Disease Modeling

Models for the study of the pathogenesis of human diseases such as cancers have depended historically on the use of animal models, especially in mice, and the limited cell resources derived from human tumors and normal tissues. Disease Modeling has recently become more feasible with the methodology of hPSCs, including human embryonic stem cells(hESCs) and induced pluripotent stem cells(iPSCs), which are derived from somatic cells of patients with or without a specific disease, by overexpression of a few transcription factors. hESCs and human iPSCs can self-renew indefinitely when cultured under the appropriate conditions, and can be differentiated into virtually any cell type. Recent advances in the induction, cultivation, and directed differentiation of hPSCs and advanced gene targeting tools provide tremendous opportunities to study carcinogenesis in many human tissue lineages. In addition, methods for inducing iPSCs provide an additional platform to model cancer in patient-specific genetic background.

Human Pluripotent Stem Cell-Based Small Cell Lung Cancer Models

In our recently published work, we have established the first method to effectively generate PNECs by directed differentiation of hPSCs (Figure 1 A). Mainly through altering NOTCH signaling, we are able to produce abundant amounts of PNEC cells (8-10% of the total culture cells) from the committed lung progenitor cells. Validated by single cell RNA (scRNA) profiling as well as other methods such as immunostaining and FACS, the hPSC-derived PNECs were physiologically functional, presented typical neuroendocrine characteristics and co- expressed almost all PNEC markers(CGRP, GRP, SYP, PGP9.5, NCAM1, Chromogranin A(CgA), NE transcription factor ASCL1[19] and lung linage marker NKX2.1) (Figure 1 B-D). The scRNA data further revealed a high similarity between the profiles of the hPSC-derived PNECs and profiles of native ones in human body(Figure 1 E).

Moreover, we demonstrated feasibility of the PNECs in modeling pulmonary neuroendocrine tumor. We made hPSC lines carrying doxycycline inducible shRNA targeting RB1 or P53, the two commonly mutated genes in SCLC. When the expression of RB was reduced specifically in the stage of differentiated lung cells, the PNEC substantially increase cell numbers and proliferation rate in culture, the typical phenotype of oncogenic transformation(Figure 1F). We also revealed a higher similarity between the profiles of our cultured cells and profiles of early stage SCLCs (stages 1a & 1b) from patients than with samples from patients with LUAD, LUSC, or neuroendocrine tumors in other organs, such as the intestine or prostate.

In addition, the PNECs with reduction of both RB and P53, when inoculated in immunodeficient mice, formed xenograft tumors that are not teratomas, and resembled early stage SCLC as characterized at morphological and molecular levels (Figure 2 A). Cells in the xenograft tumors also expressed most of the neuroendocrine biomarkers, such as CGRP, NCAM1, and ASCL1, as well as the lung lineage marker NKX2.1 (Figure 2 A). Further, the PNECs with over-expression of wide-type MYC or mutated MYC T58A in cooperation with inactivation of RB and P53, developed the advanced stage SCLC-like tumors in mice, displaying increased angiogenesis, invasion into endothelium and metastasis to liver (Figure 2 B). These findings indicated the hPSC-derived models recapitulate the main features of SCLC at various disease stages, enabling in-depth studies of SCLC in the cells with human origin and defined genetic background. **The availability of hPSC-derived PNECs also paves a new avenue for modeling and studying other types of NE tumors including pulmonary carcinoids.**

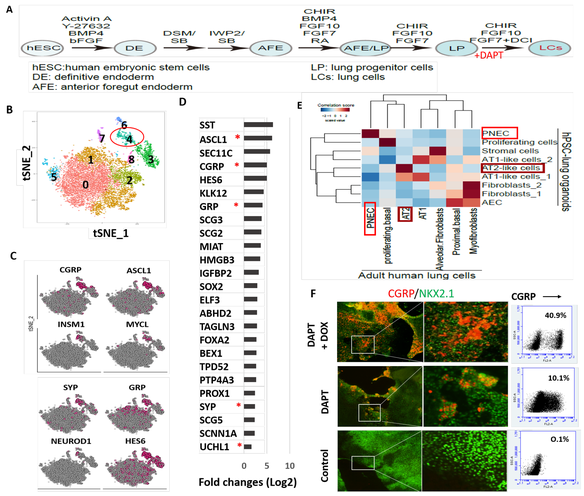


Figure 1. Generation and characterization of PNECs through directed differentiation of hPSCs. A. Scheme of directed differentiation of hESC to lung cells. **B.** scRNA profiling of lung cells derived from hESCs with NOTCH inhibition; **C.** Individual cells positive for PNECs markers and other genes associated with neuroendocrine differentiation are denoted by red dots. **D.** Putative PNEC markers differentially expressed in the PNEC-like cell cluster number 4 in panel. Bars indicate log fold-change versus non-PNEC cells. Asterisks indicate canonical PNEC markers. **E.** Correlation analysis of scRNA profiles with cell fates in hPSC-lung cells and adult human lung cells. Relative correlation level of each cluster in hPSC-lung cells with different cell types in human lung cell ranges from low (blue) to high (dark red) as indicated. **F.** DAPT treatment to inhibit NOTCH signaling effectively induced PNEC (CGRP+NKX2.1+) formation, compared to control (DMSO treatment); and loss of RB additional to inhibition of NOTCH (DAPT+DOX) significantly increased the percentage of PNECs.

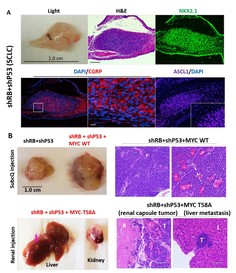


Figure 2. Lung NE tumor models generated with hPSC-derived PNECs recapitulate various stages of SCLC. **A.** hPSC-derived PNECs, induced with DOX to express shRNA specific for RB1 and P53, were subcutaneously injected into NOG mice and xenografts developed after 8-9 weeks, morphologically similar to early stage SCLC. Most cells in SCLC-like nodules are positive for the lung origin marker NKX2.1, positive for SCLC markers, CGRP and ASCL1. **B.** DAPT (blocking NOTCH signaling) treated lung cells, induced with DOX to express shRNA specific for RB1 and P53, and over-expression of MYC wild type or mutant T58A allele, were subcutaneously (upper panel) or renal capsularly (lower panel) injected into NOG mice and xenografts developed after 7-9 weeks. Both primary and metastatic tumors were morphologically similar to intermediate or late stage SCLC. (K: kidney; L: Liver; T: tumor)

Hypothesis

Although they share morphological, ultrastructural and molecular features, various pulmonary NE tumors (TC, AC, SCLC and Large cell neuroendocrine carcinoma (LCNEC)) exhibit considerably different biological characteristics and clinical behaviors. PNECs are the putative initiating cells for these types of pulmonary NE tumors, undergoing oncogenic transformation in response to specific patterns of cancer gene. Moreover, our previous study has demonstrated that the PNECs derived from hPSCs successfully transformed to SCLC-like tumors in response to the cancer genes commonly found in SCLC. This leads to the concept that the relative roles by the differentiation status of the cells undergoing transformation and by the nature of the transforming genotype together determine the characteristics of cancer. We therefore **hypothesize that the hPSC-derived PNEC can transform to pulmonary carcinoids including TC and AC, by the driver mutations specifically implicated in these tumors.** We propose the following specific aims to test the hypothesis, and to develop a new human cell-based model for pulmonary carcinoid.

Method

To establish the in vitro cell model of pulmonary carcinoid.

Among reported mutations of lung carcinoid patients, we chose oncogenes EGFR, IGF1R and tumor suppressors ARID1A and MEN1, which covers both the familial mutations and sporadic mutations to build the disease model. We will first make hPSC lines with tetracycline-inducible over-expression, shRNA to knock in, knock down or delete the candidate genes. These genetically engineered hPSC lines will then be differentiated into lung cells, and the genetic alterations will be induced only at the differentiation stage of PNECs or lung cells by tetracycline treatment. A series of experiments like counting cell numbers, calculation of mitotic index, analysis of KI67 expression and colony formation will then be performed to evaluate the proliferation, apoptosis, and oncogenic transformation of PNECs in culture. In brief, the expression level of cell cycle or mitotic division-associated genes, like KI67, TOP2A, will be measured by FACS or western blot; counting cell numbers, measuring cellular ATP levels or BrdU/EdU-based cell cycle analysis will be tested for replication and cell death rates; detection of a phosphor-histone H3 using a mitosis assay kit and calculation of mitotic index will be conducted for mitotic rate, at multiple time points to ensure positive phenotypes of oncogenic transformation can be detected. Additionally, the SCLC-like transforming PNECs with reduction of RB, P53 or over-expression of MYC will serve as a control for the above experiments.

To develop the in vivo xenograft model of pulmonary carcinoid.

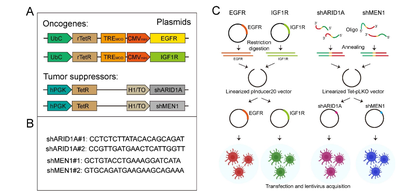
In the course of testing in vivo tumor formation with the cells generated in Aim 1, we will refine the design of the assays in various ways: a). We will first test the tumorigenesis of the culture cells by inoculating to the immunodeficient mice (e.g. the NSG strain) the whole cultures that have oncogenic potential in the types of assay in Aim 1. Since the expression vector of cancer gene encodes reporter genes (fluorescence or luciferase genes), the cells will be visibly marked to help follow their fate after introduction into mice or the tumor cells can be FACS sorted for further evaluation of their phenotypes and genotypes. b). We will also sort the cells by FACS to purify PNECs based on neuroendocrine cell surface markers (e.g. CGRP) or the fluorescence reporter protein (eYFP) to determine whether the tumor-like growths arise from PNECs; using a wide range of numbers of injected cells to determine the tumor-forming potential of the injected cells; and performing parallel experiments with the control cultures (SCLC-like transforming PNECs, or normal PNECs). c). We will also look for ways other than subcutaneous injections in mice to judge tumorigenic potential, ---the renal capsule, intra-tracheally, and intravenously---to determine growth patterns with the injection sites.

To establish the cell lines of pulmonary carcinoid.

Unlike the native pulmonary carcinoid cells in clinical samples or patient-derived xenograft (PDX) tumors that are difficult to survive and grow in in vitro culture, cancer cells within the xenografts which were originally developed from hPSC line-derived lung cultures have been tamed by in vitro culture conditions. Since our lung cultures were produced by differentiation from lung stem or progenitor cells with the capacity for long-term growth in vitro, the xenografts formed by these cells are most likely to harbor cancer stem or initiating populations, which sustain long-term proliferation and differentiation in vitro.

First, we will try to culture the cancer cells from xenograft tissue using the method for general lung cell culture. If needed, further adjustments will be applied to improve cell fitness: fetal bovine serum may be considered to add to increase viability of the cells, the medium recipes would be modified closely to the ones for pulmonary NE cell lines and various coating or embedding conditions (collagen, fibronectin or matrigel) would be tested to mimic extracellular matrix or tumor microenvironment. Once the primary cell cultures start proliferating, cellular and molecular characterization will be performed to evaluate their properties and representativeness of pulmonary carcinoid. As a supplement to existing patient-derived lines, the hPSC-derived cell lines with defined genetic background, are expected to serve as an innovative research tool for studying molecular vulnerabilities, for genetic or chemical screening and for testing drug targets of pulmonary carcinoid.

Preliminary Results



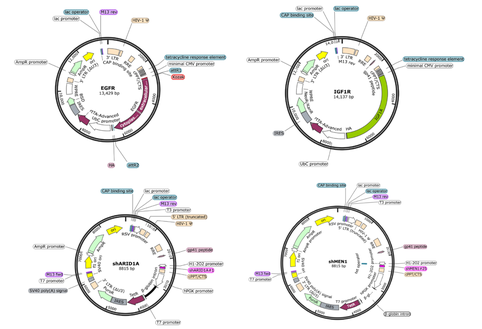


Figure 3. Lentivirus plasmid design for inducing genetic mutations

We have already designed the plasmids of the four genetic mutations that we are interested in: EGFR, IGF1R, ARID1A and MEN1. The plasmids have been transfected into hPSCs at the beginning of differentiation. Tetracycline will be added to the system to activate the expression of the plasmids after Notch inhibition to induce PNECs.

Next Step

After successfully constructing and transfecting the four plasmids and tetracycline induction, the hPSCs take a period of around 50 days to grow into lung cells with a larger proportion of PNECs carrying familial and sporadic mutations. We will characterize the generation of PNECs via PNEC and lung lineage protein markers CGRP, ASCL1 and NKX2.1. We will then characterize the mutations via both fluorescence microscope to observe the expression of fluorescent reporter proteins and western blot to check the expression level of the mutated protein. Furthermore, single-cell RNA profiling will also be a strong tool to get an accurate idea of protein expression level in the PNECs. We will then evaluate the proliferation, apoptosis and oncogenic transformation of the transfected PNECs through a series of experiments including BrdU/EdU-based cell analysis, ATP level detection, mitosis assay kit and so on.

The generation of xenograft model of pulmonary carcinoids start with the inoculation of of PNECs into immunodeficient mice to observe tumor formation after a period of time. If tumors are formed, immunohistochemistry or FACs to evaluate the molecular features of tumors including chromogranin A and neural cell adhesion molecule (NCAM1), thyroid transcription factor 1 (TTF-1), P63, S-100, CK7 and Ki67. Experts in histology will value the tumors by the number of mitoses per 10 high-power fields; the presence of necrosis; increased cellularity with disorganization; nuclear pleomorphism; hyperchromatism; and an abnormal nuclear: Cytoplasmic ratio. Single-cell RNA profiling can also be applied to get a map of expression. Finally, we will establish the cell lines of pulmonary carcinoids from the xenograft tumor we generated.

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