

# Three-Dimensional Culture and Characterization of Patient-Derived Nasopharyngeal Carcinoma Organoids

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## Primary research

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# Abstract

**Background:** Nasopharyngeal carcinoma (NPC) is a malignant tumor with high incidence in East and Southeast Asia. A proper and effective model is urgently needed to explore the mechanisms of tumorigenesis and provide new ideas and methods of cancer treatment.

**Methods:** In this study, we established a three-dimensional (3D) organoid model by patient-derived NPC tissues and adjacent tissues. We described a detailed protocol for culturing of human NPC organoids (including tissue preparation, digestion, culture, subculture, section and immunostaining). The organoids were identified by immunostaining against NPC biomarkers. The drug sensitivity against cisplatin was tested by immunostaining against Ki67 and cleaved Caspase 3.

**Results:** This method was proved to be feasible due to the presence of biomarkers including Vimentin, P63, EGFR and CK34βE12 as well as proliferative/progenitor characteristics such as Ki67, Sox2 and CD54 in NPC organoids. The chemotherapy sensitivity of NPC organoids to cisplatin treatment showed that NPC organoids may be utilized as a platform to verify the effectiveness of different antineoplastic drugs, facilitating to achieve personalized treatment.

**Conclusions:** The NPC organoid may inspire a personalized approach to the management of NPC and expand the repertoire of anti-NPC drugs.

## Background

Nasopharyngeal carcinoma (NPC) originates from epithelial lining of nasopharynx and often observed at the pharyngeal recess. Over 70% of NPC incidence is prevalent in east and southeast Asia [1, 2], and it is most commonly occurred in Southern China, where the annual incidence is about 30 cases per 100,000 persons [3]. Well-known risk factors include EBV infection, genetic susceptibility (chromosome 6p21 HLA genes, non-HLA genes) and tobacco smoking [4–6]. The untypical symptoms and preferentially metastasizing to the lymphatics causes NPC a high probability to be misdiagnosed at early stage [7, 8]. Radiotherapy is the mainstay treatment modality for non-metastatic disease at stage I. For NPC patients at other stages, chemoradiotherapy is recommended. Therefore, it is of great significance to verify the effectiveness of different antineoplastic drugs and provide patients with specific medical interventions. However, there is no reliable model to describe the pathogenesis of NPC, as well as predict therapy effectiveness and guide treatment decisions.

In vitro drug screens of 2D models have been used to characterize pathogenesis and various drug response against NPC and as a tool to understand mechanism underlying drug resistance of tumor cells [9, 10]. This model possesses economical and high-throughput screening advantages. However, 2D cell culture model cannot recapitulate the architecture and the microenvironment of NPC [11]. Results obtained from 2D cultures need to be recapitulated in *in vivo* models for further study. Currently, three-dimensional (3D) tumor organoid has been well established as a preclinical model [12] and plays an important role in screening personalized medicine [13]. These cultures are 3D structures originating from

tumor cells, which are constituted with different types of cells and forms an organ-like structure [14]. Previous studies showed that 3D culture was an ideal vehicle for revealing the pathogenesis, process and therapy of diseases. Patient-derived organoids (PDOs) have been cultured successfully in tumor of liver [15], gastric [16], colorectal [17], breast [18] and brain [19]. Researchers have also established organoids from the epithelial lining of the oral cavity, larynx, and pharynx [20]. A recent study about gastrointestinal cancers documents a strong correlation between patient clinical outcome and the response of the corresponding tumor organoids [21]. Therefore, tumor organoid can function as an innovative and reliable model in preclinical research.

Here, we established an organoid model from patient-derived NPC tissues and paracancerous tissues *in vitro*. Expression of NPC biomarkers in organoids was examined. The chemotherapy sensitivity to cisplatin was tested in NPC organoids. Thus, we provided an ideal tool to characterize the pathogenesis of NPC and to screen anti-NPC drugs, potentially providing personalized therapy to NPC patients.

## Methods

### Patient sample

Primary NPC tissues and normal adjacent tissues were collected from the patients during biopsy in Eye, Ear, Nose and Throat Hospital, Fudan University. Tissues were transferred on ice from the outpatient clinic to laboratory immediately when the biopsy examination of NPC patient was finished. Every sample was divided into two parts, and half was for pathological histology and half for 3D culture. The cultures were discarded once the pathological histology showed negative outcome. Consent forms were signed by all patients and the experimental procedures were approved by the Institutional Research Ethics Committee of Eye, Ear, Nose and Throat Hospital, Fudan University (Permit Number: 2019081).

### Culture medium preparation

The medium was sterilized through 0.22 $\mu$ m filter (Millipore, #SLGP033RB) before use. The culture medium was based on advanced DMEM/F12 (ThermoFisher, #11320033) with epidermal growth factor (EGF, 50 ng/ml, ThermoFisher, #PHG0311), basic fibroblast growth factor (bFGF, 20 ng/ml, ThermoFisher, #PHG0369), B27 (2%, Thermo Fisher, #17504044), N2 (1%, ThermoFisher, #17502001), HEPES (1 mM, ThermoFisher, #15630080), Penicillin-Streptomycin (P/S, 1%, ThermoFisher, #10378016) and Glutmax<sup>TM</sup> supplement (1%, ThermoFisher, #35050061). In addition, chemical cocktail was supplemented into the culture medium to support the favorable outgrowth of NPC organoids, including R-spondin-1 (200 ng/ml, R&D, #4645-RS), Noggin (100 ng/ml, PeproTech, #250-38), Wnt3a (50 ng/ml, R&D, #5036-WN), Y27632 (10  $\mu$ M, Sigma-Aldrich, #Y0503), A83-01 (500 nM, Sigma-Aldrich, #SML0788), N-acetylcysteine (1.25 mM, Sigma-Aldrich, #A9165) and nicotinamide (10 mM Sigma-Aldrich, #N0636). The medium was stored at 4°C and worked for one week.

### Primary nasopharyngeal carcinoma organoid culture

All the dissection apparatus was sterilized by autoclaving at 121°C for 20min. Matrigel Matrix GFR (Corning, #356231) was kept on ice until ready to use. All procedures were conducted on ice to keep the maximal viability of cultured cells. The samples were washed in ice-cold phosphate-buffered saline (PBS) for twice and sheared into small pieces. Tissues were transferred into red blood cell lysis buffer (Miltenyi Biotec, #130094183) to remove the blood cell debris and then washed in PBS. Tissue pieces were transferred to petri dishes and minced using an eye scissor into 1–2 mm<sup>3</sup> small pieces. Approximately 1~1.5 mL 0.25% trypsin-EDTA was added into tissue pieces and incubated at 37°C for 20~30 minutes to make single cell preparations. Trypsinization was stopped by addition of 0.5mL defined trypsin inhibitor (DTI, Sigma Aldrich, #T7659), and cells were centrifuged at 1,500 r.p.m. for 3 min. The supernatant was aspirated and discarded without disrupting the tumor pieces and pelleted cells at the bottom of the tube. While keeping on ice, the pellet was washed and resuspended in 1mL culture medium. The single cell suspensions were filtered by 70-µm and 40-µm nylon meshes (BD Falcon, #352350 and #352340) before seeding into Ultra-low-attached 24-well plate (Corning, #3473). Approximately 10000 cells in 0.7mL culture medium was seeded in each well. Cultures were supplemented with 3-5% (V/V) Matrigel Matrix GFR. The medium was changed every 3-4 days based on the organoid density.

### **Organoid passaging and Cisplatin treatment**

On Day14 post culture, organoids were collected by centrifuging at 1,200 r.p.m. Cell pellet were incubated with 0.25% trypsin-EDTA for 10 min at 37°C. Single cell suspension was prepared by separating the cell pellet using 1ml microsyringe. Cell suspension in culture medium was seeded in low attachment 24-well plate at density of 5000 cells in each well. For cisplatin treatment, organoids were treated with 5µM cisplatin (Sigma-Aldrich, #C2210000) for 24 hours.

### **Immunofluorescence Staining**

Organoids were collected and fixed in 4% paraformaldehyde for 15 minutes on ice. After being washed in PBS, organoids were equilibrated sequentially in 10%, 20%, and 30% sucrose, and embedded in gelatin by freezing in liquid nitrogen. The frozen organoids were cut into 20µm coronal sections on a Cryostat (Leica CM1950).

Immunofluorescence staining was performed according to a standard protocol. The organoid sections were rinsed in PBS for three times and blocked for 1 hour at room temperature in PBS containing 5% bovine serum albumin (BSA) and 0.3% Triton X-100, and followed by incubation with primary antibodies overnight at 4°C in a humidified chamber. Afterwards, the sections were rinsed three times in PBS followed by incubation with second antibodies at room temperature for 1 hour. Nucleus were counterstained with DAPI (1µg/mL, ThermoFisher, #D3571). The primary antibodies used included rabbit anti-Sox2 (#ab92494, 1:100; Abcam Inc), mouse anti-Sox2(#sc-365823, 1:100; Santa Cruz Technology), mouse anti- Ki67 (#550609, 1:100; BD Biosciences), rabbit anti-P63 (#ab63881, 1:200; Abcam Inc), goat anti-CD54 (#BAF796, 1:500; R&D), rabbit anti-Vimentin (#ab45939, 1:100; Abcam Inc), rabbit anti-E-cadherin (#ab40772, 1:500; Abcam Inc), goat anti- E-cadherin (#AF748, 1:100; R&D), mouse anti-EGFR

(#sc-120, 1:200; Santa Cruz Biotechnology), mouse anti-CK34βE12 (#m0630, 1:100; DAKO) rabbit anti-cleaved Caspase-3 (#9664, 1:100; Cell Signaling Technology). All secondary antibodies were purchased from ThermoFisher and diluted as 1:300, including Alexa Fluor 488 donkey anti-mouse (#A21202), donkey anti-goat (#A11055), donkey anti-rabbit (#A21206), Alexa Fluor 568 donkey anti-rabbit (#A10042), Alexa Fluor 594 donkey anti-rabbit (#A21207), donkey anti-goat (#A11058), Alexa Fluor 633 donkey anti-goat (#A21082) and Alexa Fluor 647 donkey anti-rabbit (#A31573). Fluorescent images were captured using Leica TCS SP8 with LAS AF Lite software.

## Statistical analysis

Organoids size was measured by SPOT software. Measurement was carried out by someone who blinded to the experimental conditions design to eliminate bias. Data were presented as mean  $\pm$  SEM from at least three independent experiments. The statistical difference was determined by unpaired t test using Graphpad Prism software and the p value less than 0.05 was considered as significant difference.

# Results

## Establishment of patient-derived NPC organoids

To propagate NPC organoids *in vitro*, we tested a range of medium composition and established a reliable protocol to maintain the growth of NPC organoids, as shown in Fig.1a. In brief, the NPC and adjacent tissues were micro-dissected from the surgical specimen to remove blood cells, digested in 0.25% Trypsin and filtered, and the resulting cell suspension was then plated in Ultra-low-attached 24-well plate. Within 3 days after plating, organoids grew out from the single cells. Visible NPC organoids were observed on Day 3 and grew steadily until Day 21 (Fig.1b-g). The organoids still maintained good status on Day 33 post *in vitro* culture (Fig.1h). Compared to the organoids derived from paracancerous tissue of patients, size of NPC organoids did not show significant difference (Fig.1i). We assessed the consistency in NPC organoid and observed similar size of organoids at passage 1 and at passage 3 (Figure.1j). Therefore, NPC organoids were well established in our defined culture medium.

## Characterization of progenitor cells in NPC organoids

To identify the stemness of the NPC organoids, we labeled them with progenitor/stem cell marker Sox2, CD54 and proliferative cell marker Ki67. We found that the organoids contained 13.2 $\pm$ 2.2% Ki67+ (Fig. 2a), 48.1 $\pm$ 11.9% CD54+ (Fig. 2c) and 69.4 $\pm$ 4.6% Sox2+ cells (Fig. 2e), demonstrating the presence of progenitor/stem cells in NPC organoids. Meanwhile, 48.17% Ki67+ cells in organoids also expressed Vimentin, a mesenchymal marker in the epithelial–mesenchymal transition (EMT) process, showing these proliferative cells were at mesenchymal state that led to the invasion and metastasis of carcinoma[22] (Fig. 2a, b, d, Ki67+/Vimentin+ cells were noted by arrowheads in a', a'', b', b'', d' and d''). Besides, 25.27% Sox2+ cells in NPC organoids also expressed P63, a biomarker of NPC [23] (Fig. 2e, g, h, Sox2+/P63+ cells were labeled by arrowheads in e', g', h'). However, these Sox2+/P63+ cells did not express E-Cadherin, indicating that these cells were not under epithelial state (Fig. 2e-h, E-

Cadherin-/Sox2+/P63+ cells were labeled by arrowheads in e'-h'). By contrast, the E-Cadherin+ cells in the NPC colonies did not express P63 (Fig. 2e-h, E-Cadherin+/P63- cells were labeled by arrows in e"-h"). These data demonstrated that the progenitor/stem cells in the NPC organoids expressed NPC biomarkers and were under mesenchymal state, potentially responsible for the tumor invasion and metastasis.

### **Organoids from NPC and paracancerous tissues show differential cellular subtypes**

To further validate the specific cellular subtypes in NPC organoids, we cultured paracancerous tissues in our established 3D culture system. Compared to the organoids from paracancerous tissue, ratio of progenitor stem cells markers Ki67 ( $p=0.7042$ ) or Sox2 ( $p=0.1682$ ) did not show apparent difference in NPC organoids (Fig. 3a-d, i). Thus, we detected whether organoids from NPC tumor and paracancerous tissue showed differential percentage of Vimentin+ and E-Cadherin+ cells. Compared to the organoids from paracancerous tissue (Fig. 3c, d), the ratio of E-Cadherin+ cells was decreased by  $14.1\pm 9.1\%$  ( $p=0.1571$ ), while the ratio of mesenchymal Vimentin+ cells was increased by  $8.9\pm 1.0\%$  ( $p=0.0001$ ) in the organoids from NPC tumor (Fig. 3a, b, i). Besides, the ratio of EGFR (a biomarker for NPC tumor[24]) positive cells, was significantly increased by  $41.9\pm 5.7\%$  ( $p=0.0001$ ) in NPC organoids than paracancerous counterparts (Fig. 3F, H, I), while the CK34 $\beta$ E12+ cell ratio in NPC organoids showed no significant difference compared to the organoids from paracancerous tissue (Fig. 3e, g, i). Furthermore,  $20.1\pm 4.2\%$  ( $p=0.0008$ ) and  $19.8\pm 14.6\%$  ( $p=0.2237$ ) more P63+ and CD54+ progenitor cells were in the NPC organoids than the cultures from paracancerous tissue (Fig. 3e-i). Therefore, NPC organoids possessed more cells under mesenchymal and stem state that was intrinsic feature in NPC but not in normal tissue.

### **NPC organoid is a potential model for drug screening**

To test the potential application in drug screening, cisplatin was added to determine the effect on NPC organoids. We observed that the presence of cisplatin led to an increase in percentage of cleaved Caspase 3+ (a marker for apoptotic cell) cells by  $19.1\pm 9.2\%$  (Fig. 4a, b,  $p=0.0393$ , cleaved Caspase 3+ cells were noted by arrowheads in Fig. 4a', b'). However, Ki67+ cells in cisplatin-treated NPC organoids did not express cleaved Caspase 3, showing that cisplatin administration did not affect these proliferative cells (Fig. 4a, Ki67+/Cleaved Caspase 3- cells were labeled by arrows in Fig. 4a'). However,  $58.4\pm 8.8\%$  Sox2+ cells underwent apoptosis with the cisplatin treatment (Fig.4b, Sox2+/cleaved Caspase 3+ cells were noted by arrowheads). In addition, we barely observed the apoptotic cells in NPC organoids without cisplatin treatment (Fig. 4c, d). This demonstrated our established NPC organoid was potentially served as an *in vitro* model for preclinical drug screening.

## **Discussion**

The majority of drug assessments for NPC were performed via 2D cell culture models [9, 10]. However, 2D models cannot recapitulate *in-vivo* cellular architecture and microenvironment [25] and limits the evaluation of drug penetration and drug resistance [11, 26]. Researchers has utilized spheroids to study drug sensitivity that generated from the C666-1 Epstein-Barr virus (EBV)-positive NPC cell line [27, 28]. Spheroids from NPC cell lines have cancer stem cell (CSC) and EMT-like characteristic. However, this

model also cannot recapitulate the conditions of tumor *in vivo*. Considering the advantages of personalized therapy against NPC, it is necessary to establish a patient-derived organoid model to investigate the inherent property of NPC and to screen the efficiency of drug therapy. High-throughput drug screen is preferentially conducted on 3D tumor cultures, considering the high economic cost and long-lasting test period when implemented on animal models [29]. A recent research verified that clinical response of seven head and neck squamous cell carcinoma patients treated with radiotherapy can be correlated to *in-vitro* responses of the corresponding organoids [20].

In this study, we present a protocol to cultivate organoids from patient-derived NPC and adjacent tissue samples that obtained from either surgical resections or biopsies. Appropriate proportion of chemical cocktail is essential for the successful establishment of NPC organoids. We found that the progenitor/stem cells (Sox2+/ CD54 + cells) in NPC organoids could expressed NPC biomarkers P63 (Fig. 2), and these cells could present mesenchymal state (Vimentin+), which relates with tumor invasion and metastasis. EMT is considered as an typical biological feature of adhesion loss and metastatic potential acquisition in tumor cells [30]. The characteristics of Vimentin and E-Cadherin expression on organoids derived from NPC tumor and paracancerous tissue was similar with that of primary tumors and normal tissues [31]. Therefore, it is suggested that NPC organoids may be composed of a high proportion of mesenchymal cells and stem state, which is an intrinsic feature of NPC cells but not normal tissues.

Overexpression of EGFR in NPC is an important mechanism for post-therapeutic recurrence, metastasis and poor survival of patients [32, 33]. The cancer stem cells (CSCs) theory implies that the stemness-associated genes are correlated with abnormal capability of self-renewal and generating new tumor cells, which enhance progression of EMT [34, 35]. Successful establishment of NPC organoids was verified in our study by immunostaining against tumor biomarkers such as EGFR and Vimentin (Figs. 2 and 3). Overexpressed EGFR is found in NPC organoids which is consistent with the reports that approximately 85% of NPC patients in China are with a high-level expression of EGFR [32, 36]. Vimentin and E-Cadherin are crucial EMT-related CSC biomarkers [37] and the overexpression of Vimentin was confirmed in NPC organoids, demonstrating a potential tendency to tumorigenesis (Fig. 3). Therefore, we established a 3D model of patient-derived NPC tumors, and performed cisplatin screen to explore the feasibility of testing the sensitivity of drugs against NPC *in vitro*. Further researches are needed to check the validity of this NPC organoid as valuable pre-clinical model for academic, clinical, and pharmaceutical research.

## Conclusion

In this study, we establish organoid of nasopharyngeal carcinoma via patient-derived tumor tissues. These organoids express NPC biomarkers, potentially functioning as an ideal platform for the accurate and economic preclinical drug screening, which is essential for the personalized medicine research in NPC patients.

## Abbreviations

NPC: Nasopharyngeal carcinoma, 3D: Three-dimensional, PDOs: Patient-derived organoids, EBV: Epstein-Barr virus, CSC: cancer stem cell, EMT: Epithelial–mesenchymal transition.

## Declarations

### Ethics approval and consent to participate

The experimental procedures were approved by the Institutional Research Ethics Committee of Eye, Ear, Nose and Throat Hospital, Fudan University (Permit Number: 2019081).

### Consent for publication

Not applicable.

### Availability of data and materials

All data generated or analyzed during this study are included in this published article.

### Competing interests

The authors declare that they have no competing interests.

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### Authors' contributions

LW, XZ, XF, CD, YY performed experiments. LW, XZ, HY, YY analyzed data. JL and BZ collected samples. LW, XZ, HY, YY wrote the manuscript. All authors read and approved the final manuscript.

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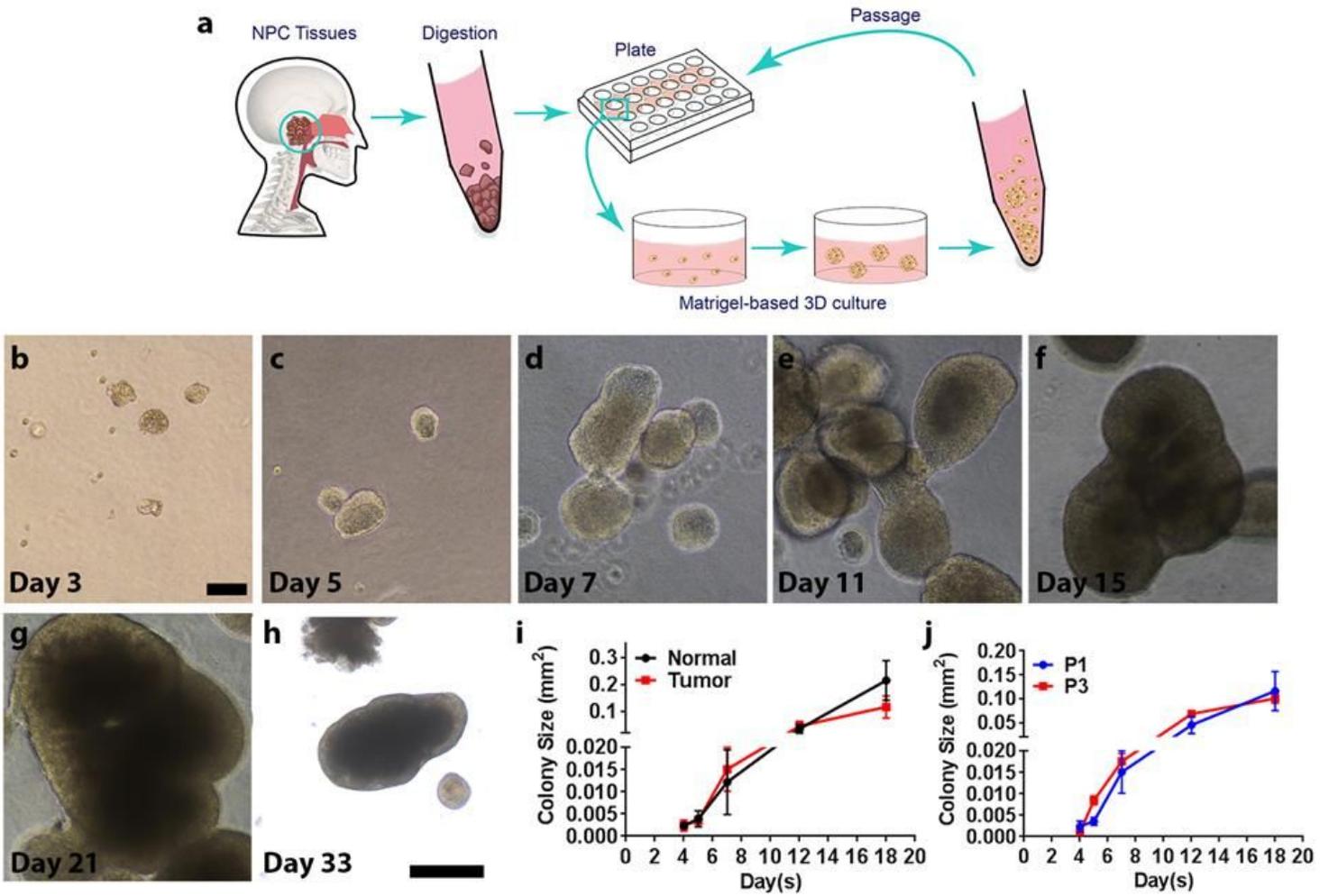
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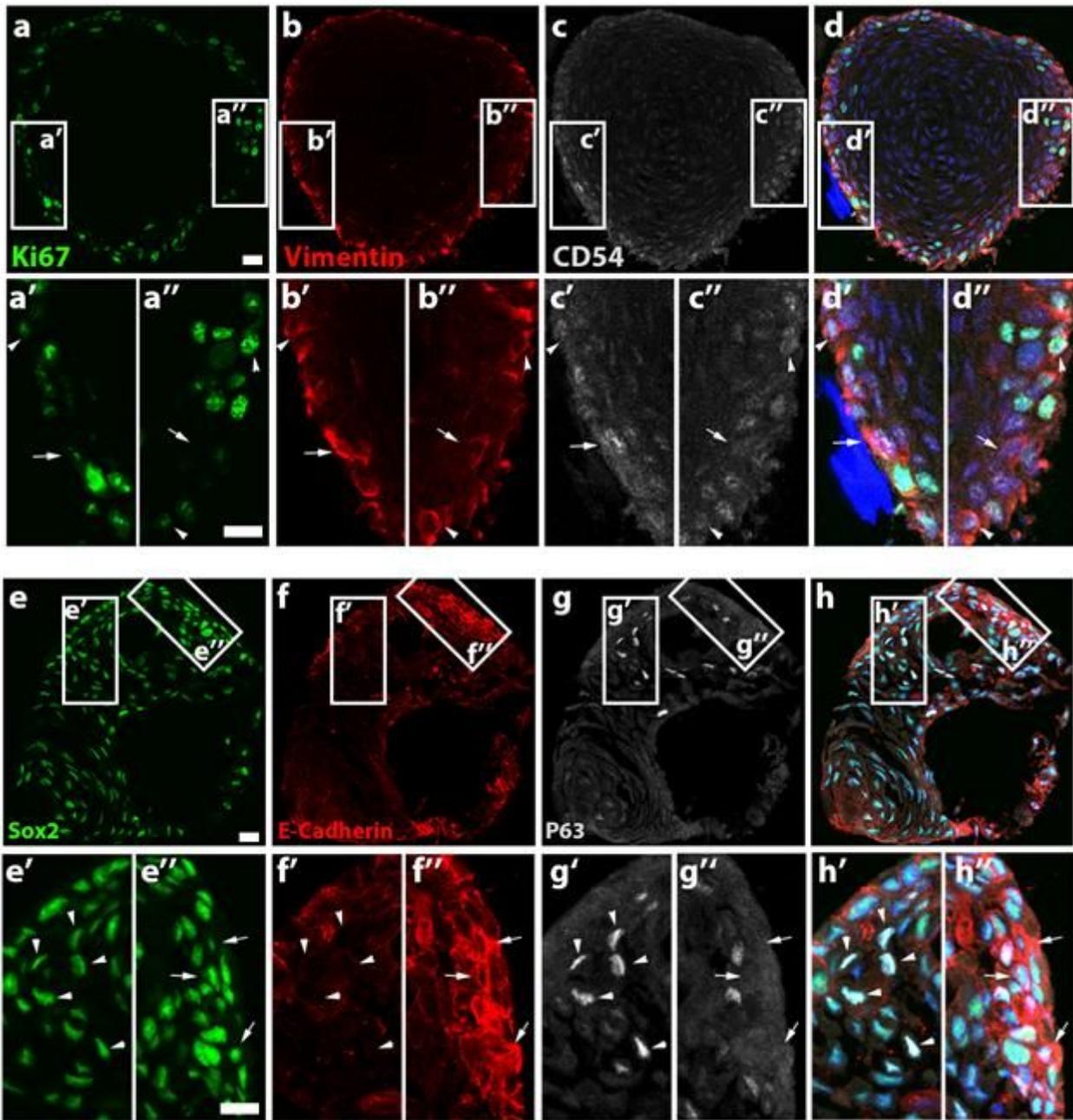
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## Figures



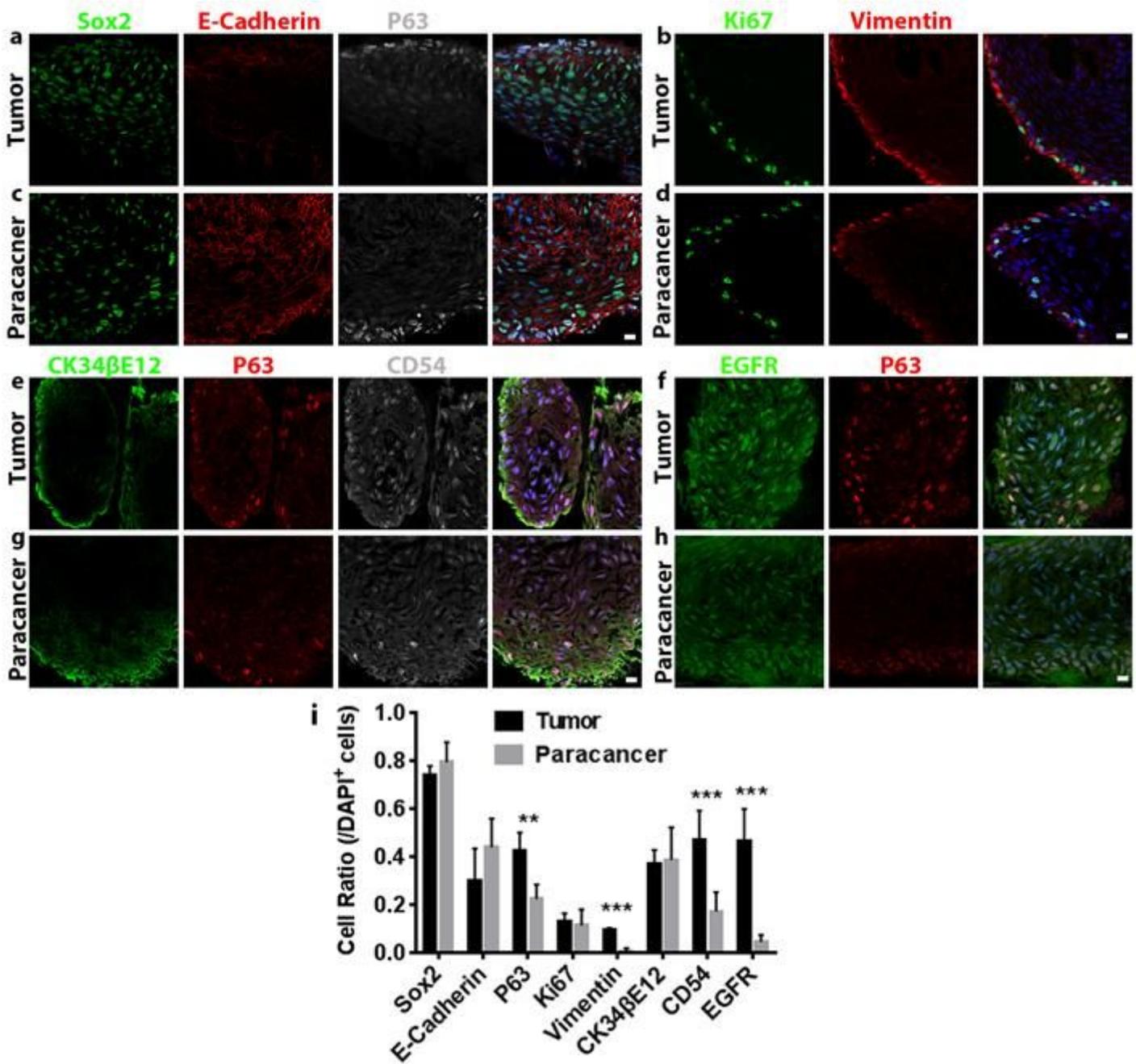
**Figure 1**

Culture of patient-derived NPC organoid. (a) Schematic protocol of NPC organoid culture. (b-h) Microscopic images of NPC organoids on Day 3 to Day 33 post in vitro culture. (i) Statistical analysis on size of organoids from NPC and paracancerous tissue. (j) Statistical analysis on size of organoids at P1 and P3. Scale bars were 100 μm in (b) and 500μm in (h).



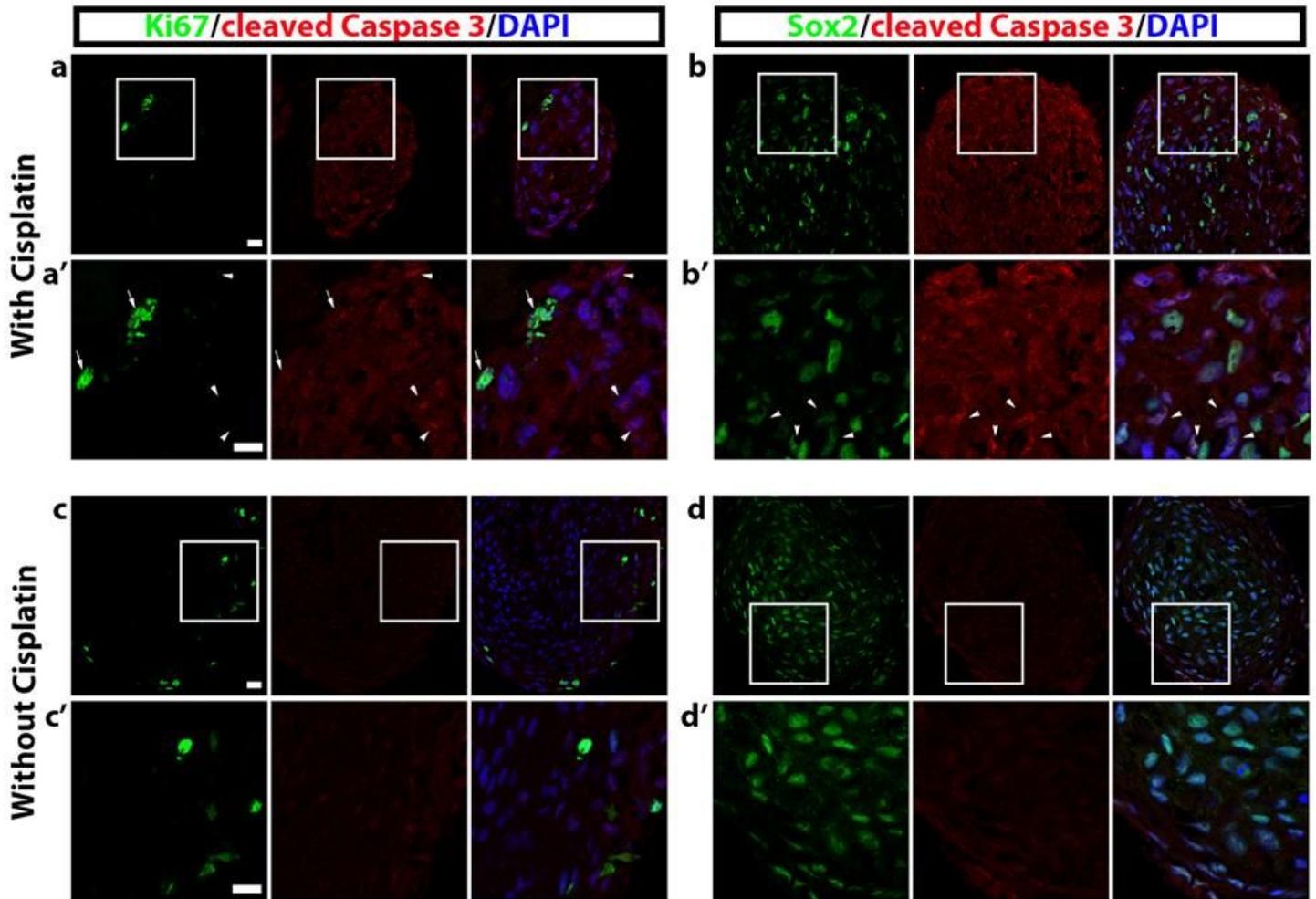
**Figure 2**

Characterization of progenitor/stem cells and biomarker expression in NPC organoids. (a-d) Confocal images of Ki67<sup>+</sup>, Vimentin<sup>+</sup> and CD54<sup>+</sup> cells in NPC organoids. The squares in (a-d) were shown as (a'-d', a''-d''). Ki67<sup>+</sup>/Vimentin<sup>+</sup>/CD54<sup>+</sup> cells were noted by arrowheads while Ki67<sup>-</sup>/Vimentin<sup>+</sup>/CD54<sup>+</sup> cells were labeled by arrows. (e-h) Immunostaining against Sox2, E-Cadherin and P63 in NPC organoids. The boxed regions in (e-h) were highlighted in (e'-h', e''-h''). Sox2<sup>+</sup>/E-Cadherin<sup>-</sup>/P63<sup>+</sup> cells were noted by arrowheads while E-Cadherin<sup>+</sup>/P63<sup>-</sup> cells were labeled by arrows. Scale bars were 20  $\mu$ m.



**Figure 3**

Difference in cellular subtypes in organoids derived from NPC and paracancerous tissue. (a-d) Confocal images of Sox2+, E-Cadherin+, P63+, Ki67+ and Vimentin+ cells in NPC organoids (a, b) and cultures from paracancerous tissue (c, d). (e-h) Immunostaining against CK34βE12, P63, CD54 and EGFR in organoids from NPC (e, f) and paracancerous tissue (g, h). (i) Statistical analysis on the ratio of positively-stained cells. The statistical significance was determined by unpaired ttest Scale bars were 20 μm.



**Figure 4**

Cisplatin treatment induced cell death in NPC organoids. (a-d) Immunostaining against Ki67, cleaved Caspase 3 and Sox2 in cisplatin-treated (a, b) and untreated NPC organoids (c, d). Squared regions in (a-d) were shown as (a'-d'). In (a'), Ki67+/cleaved Caspase 3- cells were labeled by arrows while Ki67-/cleaved Caspase 3+ cells were noted by arrowheads. In (b'), Sox2+/cleaved Caspase 3+ cells were labeled by arrowheads. Scale bars were 20  $\mu$ m.