Supplemental of:

3D culture model to distinguish normal from malignant human bronchial epithelial cells

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SUPPLEMENTAL MATERIAL AND METHODS

Cell Culture

Primary human normal bronchial epithelial cells (HBEC) were obtained from Clonetics Cell Systems (Lonza Ltd, Basel, Switzerland) and cultured in bronchial epithelial growth cell medium (BEGM) (Lonza Ltd) supplemented with 5ng/ml EGF. All experiments were carried out using cells from passages 1 to 4. We used five bronchial epithelial lung cancer cell lines: A549, H1838, H23, H460, CaluI (ATCC). These cells were cultured in DMEM (supplemented with 10% foetal bovine serum (FBS) and 1% penicillin/streptomycin mixture), except for H1838, H460 and CaluI, which was cultured in RPMI (supplemented with 10% FBS and 1% penicillin/streptomycin mixture). Cells were grown into monolayer culture (two-dimensional culture, 2D) or into laminin-rich basement membrane growth factor reduced Matrigel (BDBiosciences) (Matrigel) as single cells in 8-well chamber slide designed for confocal microscopy experiments (BD) (three-dimensional culture, 3D). The Matrigel protein concentration was 7.8-9.5 mg/ml. Matrigel Matrix Growth Factor Reduced is a solubilized basement membrane preparation extracted from the Engelbreth-Holm-Swarm (EHS) mouse sarcoma, a tumor rich in extracellular matrix proteins. Its major component is laminin, followed by collagen IV, heparan sulfate proteoglycans, entactin/nidogen. . The 3D culture was setup with

the embedded method based on Wu *et al.* . A lower layer of matrix was gelled before adding the cell/matrix suspension, to avoid sphere contact with plastic. The medium was changed every second or third day and cultures were kept in a humidified atmosphere of 5% CO2/95% O2.

Immunofluorescence and image acquisition

Structures were prepared as previously described (Lee *et al.*, 2007). All samples were imaged through the bottom of 8-well chamber slides designed especially for use with confocal microscopy. Sections were stained for: β-catenin, p63, Mucin 5B (MUC5B), lysozyme (AZGP1) and β-tubulin IV. Secondary antibody conjugated either to Alexa-488 or Alexa-546 was used. Negative control sections were stained with primary or second antibodies alone. Apoptosis was assayed by immuno-detection of activated caspase 3 with conjugated Alexa-488 antibody. Nuclei were stained with DAPI. A minimum of 100 acinar structures were counted per experiment, and each experiment was performed at least three independent times. For 2D staining, cells were directly fixed using 4% paraformaldehyde, and samples were incubated with primary antibody followed by either Alexa-Fluor-488 or Alexa-Fluor-546-conjugated secondary antibody. Nuclei were counterstained with DAPI. Confocal analysis was performed by using the Nikon confocal imaging system. Images were generated, converted to Tiff format and arranged by using Adobe Photoshop 7.0. The size and the number of acini were measured based on the methods of Wu *et al.*. Briefly, the number and diameter of each acini picture was measured in μms using the EZ.C1 3.9 Software (Nikon). The mean acini was then determined for each sets of acini using GraphPad Prism Software.

Clinical Specimens.

All subjects gave their written informed consent to participate in the study, after the nature of the procedure had been fully explained. The study followed recommendations outlined in the Helsinki Declaration and received approval from the Local Research Ethics Committee. Specimens for culture were obtained from patients with a confirmed histological diagnosis of NSCLC at the time patients were undergoing approved protocol-staging procedures and included surgically resected tumor masses.

Primary human Bronchial epithelial cell culture from lung explants

Solid tumor specimens were obtained immediately after surgery. For culturing primary cells from lung explants, we used Yaghi *et al.* method (Yaghi et al., 2010) with minor modifications. Briefly, bronchial segments are rinsed in HBSS (PAA) and cut open and minced into 2-3 mm pieces of tissue. After coating 100 mm culture plates for at least 1 hour with a combination of collagen (30 µg/ml), fibronectin (10 µg/ml), and BSA (10 µg:ml), the plates are scratched in 5 areas and tissue pieces are placed, the culture medium (BEGM) is added and plates are placed in the incubator at 37°C in 5% CO2. The culture medium is changed every 4 days. The epithelial cells grow in about 4 weeks. Cells are then trypsynized and seed in T25 flask. Once cells are confluent enough, cells are grown in 3D Matrigel for ten days as described above.

SUPPLEMENTAL TEXT

Influence of the medium on spheres formation

To measure the influence of the medium on spheres formation, we have grown in 3D, A549 cells and compare the formation of the sphere in both type of medium DMEM (which is the specific medium for growing A549 cells) (fig. S3a) to BEGM medium form Lonza (which is the specific medium for growing HEBC cells) (fig. S3b). As shown in fig. S3b, A549 did not grow in 3D in BEGM medium from Lonza. Therefore, the BEGM medium which has more growths factor does not favour the formation of A549 cancer sphere cells.

SUPPLEMENTAL FIGURES

SUPPLEMENTAL FIGURE S1. Cellular expression of β-catenin and p63 in 2D culture. HBEC and A549 cells were grown on coverslips. Cells were then fixed with paraformaldehyde and proceed for immunofluorescence with an antibody against β-catenin (a) or p63 (b) and DAPI for nuclear staining. Scale Bar 50 µm. Images were acquired by confocal microscopy on Nikon Eclipse 2000-C1.

SUPPLEMENTAL FIGURE S2. Cellular expression of mucin 5B (MUC5B), lysozyme (AZGP1) and β-tubulin IV in 2D-monolayer. HBEC and A549 cells were grown on coverslips. Cells were then fixed with paraformaldehyde and proceed for immunofluorescence with an antibody against MUC5B (a), AZGP1 (b) or β-tubulin IV (c) and DAPI for nuclear staining. Scale Bar 50 μm. Images were acquired by confocal microscopy on Nikon Eclipse 2000-C1.

SUPPLEMENTAL FIGURE S3. Influence of growth medium on spheres formation in A549 3D culture. Phase contrast of A549 grown in 3D culture in complete DMEM medium (a) as compared to A549 grown with complete BEGM medium (HBEC medium) (b) at day 10 in 3D Matrigel.

SUPPLEMENTAL REFERENCES

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