SOX2 Drives Bronchial Dysplasia in a Novel Organotypic Model of Early Human Squamous Lung Cancer

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Abstract

Rationale: Improving the early detection and chemoprevention of lung cancer are key to improving outcomes. The pathobiology of early squamous lung cancer is poorly understood. We have shown that amplification of sex-determining region Y-box 2 (SOX2) is an early and consistent event in the pathogenesis of this disease, but its functional oncogenic potential remains uncertain. We tested the impact of deregulated SOX2 expression in a novel organotypic system that recreates the molecular and microenvironmental context in which squamous carcinogenesis occurs.

Objectives: (1) To develop an *in vitro* model of bronchial dysplasia that recapitulates key molecular and phenotypic characteristics of the human disease; (2) to test the hypothesis that SOX2 deregulation is a key early event in the pathogenesis of bronchial dysplasia; and (3) to use the model for studies on pathogenesis and chemoprevention.

Methods: We engineered the inducible activation of oncogenes in immortalized bronchial epithelial cells. We used three-dimensional tissue culture to build an organotypic model of bronchial dysplasia.

Measurements and Main Results: We recapitulated human bronchial dysplasia *in vitro*. SOX2 deregulation drives dysplasia, and loss of tumor promoter 53 is a cooperating genetic event that potentiates the dysplastic phenotype. Deregulated SOX2 alters critical genes implicated in hallmarks of cancer progression. Targeted inhibition of AKT prevents the initiation of the dysplastic phenotype.

Conclusions: In the appropriate genetic and microenvironmental context, acute deregulation of SOX2 drives bronchial dysplasia. This confirms its oncogenic potential in human cells and affords novel insights into the impact of SOX2 deregulation. This model can be used to test therapeutic agents aimed at chemoprevention.

Keywords: early lung cancer; sex-determining region Y-box 2 (SOX2); bronchial dysplasia; squamous lung cancer; organotypic culture

(Received in original form October 27, 2015; accepted in final form February 15, 2017)

Supported by the Wellcome Trust, and by Wellcome Trust Intermediate Clinical Fellowship WT097143MA (F.M.M.), the Roy Castle Lung Cancer Foundation (F.M.M.), Cancer Research UK grants C4750/A12077 and C4750/A19013 (T.D.L. and G.I.E.), the National Institute for Health Research (NIHR) Biomedical Research Centre based at Guy's and St. Thomas' National Health Service Foundation Trust and King's College London, by Medical Research Council Program Grant G1100238 (P.L.), and, in part, by NIHR Biomedical Research Centre in Cambridge and the Cancer Research UK Cambridge Centre (R.C.R. and D.M.R.).

The views expressed are those of the authors and not necessarily those of the National Health Service, the National Institute for Health Research, or the Department of Health.

Author Contributions: L.L.C. performed most of the experimental work; F.M.M. conceived the study, performed some of the experimental work, and wrote the manuscript; J.-A.J. performed some experimental work; H.F. and T.S. contributed protocol advice; D.M.R. and R.C.R. contributed the tissue microarray; D.M.R. reviewed histology; P.M., P.L., and J.B. contributed to study design and bioinformatic analyses; E.L.R., G.I.E., and T.D.L. contributed to study conception and design; L.L.C., R.C.R., J.-A.J., E.L.R., T.D.L., and G.I.E. helped with editing the manuscript.

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This article has an online supplement, which is accessible from this issue's table of contents at www.atsjournals.org

Am J Respir Crit Care Med Vol 195, Iss 11, pp 1494–1508, Jun 1, 2017

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Originally Published in Press as DOI: 10.1164/rccm.201510-2084OC on February 15, 2017

Internet address: www.atsjournals.org

At a Glance Commentary

Scientific Knowledge on the

Subject: Sex-determining region Y-box 2 (SOX2) is amplified in high-grade bronchial dysplasia, a preinvasive stage of squamous lung cancer (SQC), as well as in SQC. However, the role and impact of SOX2 amplification is uncertain. There are no *in vitro* models of preinvasive SQC that faithfully recapitulate the human disease.

What This Study Adds to the

Field: Here, we show that SOX2 activation—in a three-dimensional culture system at the air–liquid interface, and in the appropriate molecular context that recapitulates the human disease—is sufficient to drive the dysplastic phenotype. This implicates SOX2 deregulation as a key early event in squamous carcinogenesis. We identify critical downstream sequelae of SOX2 deregulation, and show that this model has broad utility for understanding the molecular pathogenesis of early disease and assaying the impact of chemopreventive agents.

Lung cancer is one of the most common causes of cancer-related mortality in the world (1). Squamous lung cancer (SQC) accounts for approximately 30% of all lung cancers. There are a number of challenges to reducing the mortality from SQC, and these include improving smoking cessation, early detection and diagnosis, and the development of novel approaches to the chemoprevention and treatment of lung cancer (2–4).

A key aspiration is to understand the molecular pathogenesis of SQC and to use this information to drive developments in early detection, chemoprevention, and treatment (2). This would be significantly facilitated by model systems that faithfully recapitulate the human disease. There has recently been progress in the development of murine models of SQC, and these models will lead to new insights (5–7). However, there are advantages to *in vitro* models, including the use of human cells, genetic tractability, throughput, costs, and reduction in animal use.

The epithelial cell–microenvironment interaction is critical to the pathogenesis of

epithelial malignancies, and to resistance to therapeutics (8). It has been repeatedly demonstrated that cancer cells behave differently and have very different phenotypic outcomes in complex compared with traditional culture systems (9). Advances in tissue engineering have made it possible to use *in vitro* models of the bronchial wall to investigate pathological mechanisms in asthma, chronic obstructive pulmonary disease, and cancer (10, 11). In the current study, we have built on these studies to build a rational model of bronchial dysplasia, the precursor lesion to SQC.

The key molecular drivers in the early pathogenesis of SQC remain obscure. In a series of reports on a small number of patients, it has been demonstrated that loss or mutation of p53 is an early event (12, 13) that appears to afford a clonal survival and expansion benefit, even at the earliest histopathological stages. Furthermore, the tumor promoter 53 gene (*TP53*) is disrupted in almost all invasive SQCs (14).

It has repeatedly been demonstrated that 3q amplification occurs before invasion in the pathogenesis of SQC (15). In previous work, we have shown that 3q amplification marks the progression between low- and high-grade dysplasia, and has implications for prognosis (16). This has been confirmed in a larger cohort (17). It is likely that multiple genes on 3q can contribute to squamous carcinogenesis (18). However, previous mapping studies, both in SQC and bronchial dysplasia, have identified sex-determining region Y-box 2 (SOX2) as a likely key target of the 3q amplicon (16, 19, 20).

Therefore, the available molecular epidemiological evidence suggest that p53 is disrupted early in the pathogenesis of the disease, and that 3q amplification and associated deregulation of SOX2 expression occurs at the epidemiological and histological bottleneck between low- and high-grade bronchial dysplasia (16, 17, 21).

SOX2 is a single-exon nuclear transcription factor. It is pleiotropic, with critical roles in stem cell and developmental biology, and it is particularly important in the development of the lung (22, 23). There have been a number of reports regarding the potential for SOX2 overexpression to drive carcinogenesis in murine models. In one report, deregulated SOX2 was unable to drive carcinogenesis when its overexpression was restricted to Club cells using the secretoglobulin family 1A member 1 (*Scgb1a1*) promoter (24). In a separate report, marked

overexpression was sufficient to form phenotypic adenocarcinomas that were positive for p63, a squamous carcinoma marker (25). More recently, the delivery of intratracheal lentivirus containing SOX2 in conjunction with loss of liver kinase B1 or serine/threonine kinase 11 was sufficient to drive squamous carcinogenesis, albeit at relatively low penetrance (6). While this article has been in press a further murine model of squamous lung cancer was reported demonstrating that SOX2 overexpression, when combined with naphthalene-induced epithelial injury and loss of key tumor suppressors, acts as an oncogenic switch in multiple cell lineages in the mouse lung (26).

In vitro experiments using immortalized human cell lines have given varying results. Overexpression of SOX2 was sufficient to transform SV40 large T antigen immortalized BEAS2B cells (20); virally immortalized small airway epithelial cells were transformed by SOX2, but only in conjunction with cooperating genetic lesions (19).

We have modeled the earliest stages of bronchial epithelial carcinogenesis using nonvirally immortalized human bronchial epithelial cells (HBECs) with a stable karyotype and an intact p53 signaling response/pathway (27). Using lentiviral constructs, we sequentially knock down TP53 and inducibly deregulate SOX2 expression. We establish short-term organotypic cultures (OTCs) and demonstrate that SOX2 deregulation, on a background of p53 knockdown in a confluent epithelial monolayer at the air–liquid interface (ALI), drives the dysplastic phenotype *in vitro*. We show that SOX2 alters key cell signaling pathways, recapitulating signatures seen in invasive SQC, and that it deregulates multiple genes implicated in hallmarks of cancer progression, including inhibition of apoptosis, driving cell proliferation, and cell migration and epithelial–mesenchymal transition (EMT). We demonstrate that the model can be used to test chemoprevention strategies, and that a specific inhibitor of AKT that is already in early-phase trials prevents the development of the dysplastic phenotype. This work establishes that SOX2, in the appropriate molecular and environmental context, is sufficient to drive lung squamous carcinogenesis in human cells and establishes a novel model of human bronchial carcinogenesis with broad utility.

This work has been presented, in part, in abstract form at a number of scientific meetings (28–30).

Methods

Cell Culture

HBECs immortalized with human telomerase reverse transcriptase (hTERT) and CDK4 (hereafter called KT cells) were kindly provided by J. W. Shay and J. D. Minna (University of Texas Southwestern Medical Center, Dallas, TX) (27). The following plasmids were used to generate lentiviral particles using standard protocols—FUW-M2rtTA and FUW tetO-lox-hSOX2 (31), pLVUshp53-tTR-KRAB, and pLVU-tTR-KRAB (32). KT cells were sequentially transduced with lentiviral particles to create a suite of cell lines with different genotypes. Cell lines were cultured in keratinocyte serum-free medium supplemented with human recombinant epidermal growth factor and bovine pituitary extract (Life Technology, Paisley, UK) and bronchial epithelial basal medium (Lonza, Basel, Switzerland). A549 cells with a tetracycline-inducible red fluorescent protein reporter were maintained in Dulbecco's modified Eagle medium (DMEM)/10% fetal bovine serum (FBS).

OTC

OTCs were established in 12-well Transwell plates with a porous membrane insert (pore size, 0.4 μ m; Corning 3460; Corning, Wiesbaden, Germany). On Day 1, a mixture of collagen (Corning Collagen I, Rat Tail), 10x DMEM, 10% tetracycline low FBS, 7.5% sodium bicarbonate, and MRC-5 fibroblasts (final concentration 10^4 cells/ml) was prepared on ice. Prior to adding the fibroblasts the pH was adjusted to 7.25–7.3 with 1 M sodium hydroxide. This solution was inverted gently to obtain a homogenous solution without bubbles and 600 μ l of the mixture poured into each Transwell. Plates were incubated at 37°C for 30 minutes and medium (keratinocyte serum-free medium plus supplements) was added to both the upper and lower chambers. The fibroblasts were left to contract the collagen for 5 days, and HBECs were then overlaid on the collagen layer. HBECs were kept covered until they reached confluence, and on Day 8 an ALI was generated through the removal of medium from the upper chamber. Medium with or without 2–5 μ g/ml doxycycline supplementation in the lower chamber was changed every 48 hours and samples fixed on or after Day 14 with 10% formaldehyde at 4°C before further processing; 4.5- μ m sections were prepared for immunofluorescence or

immunohistochemistry using standard protocols. The following antibodies and dilutions were used: SOX2 (cat. no. MAB2018, 1:200 dilution; R&D Systems, Abingdon, UK), marker of proliferation Ki67 (Ki67) (cat. no. RM-9106-S1, 1:200 dilution; Thermo Scientific, Fremont, CA), p-AKT (Ser473; cat. no. 3787, 1:50 dilution; Cell Signaling Technology, Danvers, MA), phospho-extracellular signal-regulated kinase (pERK) 1/2 (cat. no. 4376, 1:1,000 dilution; Cell Signaling Technology), and acetylated tubulin (cat. no. T7451, 1:1,000 dilution; Sigma, St. Louis, MO). For chemoprevention studies, AZD5363 was dissolved in dimethyl sulfoxide and added to the lower chamber to a final concentration of 10 μ M at the same time that doxycycline was added.

Differentiation culture. The 24-well Transwells (cat. no. 353095; Corning) were coated with collagen. Up to one hundred thousand cells were plated onto each Transwell and submerged in 200 μ l of Complete PneumaCult expansion medium (cat. no. 05008; Stem Cell Technologies, Vancouver, Canada) plus penicillin/streptomycin, with 500 μ l in the lower chamber. On Day 5, an ALI was generated by the removal of the medium from the upper chamber; 500 μ l of PneumaCult ALI Maintenance medium plus penicillin/streptomycin was added to the lower chamber (cat. no. 05001; Stem Cell Technologies) and cells were washed with phosphate-buffered saline (PBS) and medium changed every 3 days; 23-day ALI cultures were then fixed with 4% paraformaldehyde for 10 minutes at room temperature. Membranes were cut from the Transwells and cells permeabilized with PBS plus 0.3% Triton for 15 min followed by blocking with PBS plus 0.1% Triton plus 5% normal goat serum plus 1% bovine serum albumin for 45 minutes. Membranes were then incubated with the following antibodies and dilutions: keratin 5 (K5; cat. no. PRB-160P, 1:500 dilution; Covance, Princeton, NJ), mucin 5AC (cat. no. MS-145P0, 1:200 dilution; Thermo Scientific), γ -tubulin (cat. no. SAB4503045, 1:200 dilution; Sigma), acetylated tubulin (cat. no. T7451, 1:1,000 dilution; Sigma), and CC10 (cat. no. sc25555, 1:500 dilution; Santa Cruz Biotechnology, Dallas, TX). Nuclei were stained using Hoechst 33,258 dye (Sigma), and samples were mounted in Fluoromount Aqueous Mounting Medium (Sigma) and analyzed using an AxioImager compound microscope (Carl Zeiss, Cambridge, UK).

Western Blotting

Protein extracts were obtained using RIPA buffer; 40 μ g of protein was loaded onto 4–12% tris-acrylamide gels followed by transfer to a PVDF membrane (Millipore, Billerica, MA). The following antibodies were used: SOX2 (cat. no. MAB2018, 1:1,000 dilution; R&D Systems), p53 (cat. no. sc-126, 1:1,000 dilution; Santa Cruz Biotechnology), K5 (cat. no. ab24647, 1:1,000 dilution; Abcam), p63 (cat. no. ab735, 1:1,000 dilution; Abcam), and β -actin (cat. no. sc-47778, 1:5,000 dilution; Santa Cruz Biotechnology). Blotting results were detected by an enhanced chemiluminescence kit (Thermo Scientific) before exposing to X-ray film (Fujifilm, Tokyo, Japan).

Cell Culture Assays and Cell Cycle Analysis

For cell growth assays in standard culture, 0.1×10^5 cells from each cell line were seeded with or without 1 μ g/ml doxycycline. Cells were trypsinized at specified time points and counted using the Countess Automated Cell Counter (ThermoFisher Scientific, Paisley, UK). For cell cycle analysis, 0.1×10^6 cells were plated in a six-well plate. For three-dimensional (3D) cultures, to harvest the cells at the ALI, medium was removed from both the lower and upper chamber and the porous membrane of each Transwell wrapped in cling film. Collagen discs were washed twice with PBS and treated with trypsin for 12 minutes at 37°C. Cells were collected in DMEM/10% FBS and the pellet resuspended in PBS/5 mM ethylenediaminetetraacetic acid. Cells were fixed and stained with propidium iodide. Samples were analyzed on an Accuri C6 (BD Biosciences, Oxford, UK) and further analysis was performed using FlowJo software (FlowJo LLC, Ashland, OR).

Tissue Microarray

A tissue microarray was constructed using surgical and endobronchial patient biopsy specimens from Papworth Hospital National Health Service Trust (Cambridge, UK). Biopsies assessed by a consultant thoracic histopathologist to be high-grade bronchial dysplasia were used for this study (limited to one core per individual patient).

Chromatin Immunoprecipitation–Polymerase Chain Reaction

Bronchial epithelial cells were cultured in the OTC system and untreated/treated with 2 μ g/ml doxycycline for 8 days before being

trypsinized and harvested. Cells were processed using the Magna Chromatin Immunoprecipitation (ChIP) G–ChIP kit (Millipore), per the manufacturer's instructions. Chromatin shearing was performed using Covaris S2 series and the following program: duty cycle, 2%; intensity, 3; cycles per burst, 200, for 12 minutes. Sheared chromatin (50 μ l) was incubated with magnetic beads and the following antibodies: SOX2 (cat. no. AF2018, 5 μ g; R&D Systems) and IgG control (cat. no. AB-108-C, 5 μ g; R&D Systems). Samples were validated using the manufacturer's recommended antibody followed by polymerase chain reaction (PCR) of the eluted DNA (ChIPab⁺ RNA Pol II–ChIP Validated Antibody and Primer set; Millipore).

RNA extraction and quantitative PCR. RNA was extracted from cells cultured in 3D, untreated/treated with 2 μ g/ml doxycycline for 8 days, using the miRNeasy Mini Kit (Qiagen, Manchester, UK). Collagen disks were solubilized with 1 ml of QIAzol Lysis Reagent (Qiagen) by vortexing and RNA was isolated following manufacturer's instructions. RNA was eluted with RNase-free water and

treated with DNase I (ThermoFisher Scientific). Complementary DNA (cDNA) was prepared with 1 μ g of total RNA using the High Capacity cDNA Reverse Transcription Kit (ThermoFisher Scientific). Quantitative PCR was performed using SYBR green reagents and glyceraldehyde phosphate dehydrogenase as internal control. Thermal cycling conditions were as follows: 95°C for 3 minutes, 40 cycles at 95°C for 15 seconds, and 55°C for 30 seconds. The relative amount of messenger RNA in each sample was quantified using the $\Delta\Delta$ Ct method.

RNA sequencing. Sample sequencing libraries were prepared from 200 ng of total RNA extracted from treated and control OTCs 4 days after the addition of doxycycline. The TruSeq Stranded mRNA HT sample preparation kit (Illumina, Chesterford, UK) was used according to the manufacturer's instructions. Samples were individually indexed for pooling using a dual index strategy. Libraries were quantified on a TapeStation DNA 1,000 Screen tape (Agilent, Cheshire, UK) and by quantitative PCR (qPCR) using a next-generation sequencing (NGS) Library Quantification Kit (KAPA

Biosystems, London, UK) on an AriaMx qPCR system (Agilent). Libraries were then normalized, pooled, diluted, and denatured for sequencing on the NextSeq 500 (Illumina) according to the manufacturer's instructions. Samples were pooled such that a minimum of 25 M unique clusters per sample was achieved. PhiX control library (Illumina) was spiked into the main library pool at 1% vol/vol for quality control purposes. Sequencing was performed using a high-output flow cell with 2 $\times$ 75 cycles of sequencing providing 800 M paired end reads from 400 M unique clusters.

RNA-seq was performed and analyzed as a service by Cambridge Genomic Services (Department of Pathology, University of Cambridge, Cambridge, UK). RNA was sequenced (2 $\times$ 75 bp) using the Illumina NextSeq500. The quality control of the reads was evaluated using FastQC (Babraham Bioinformatics, Cambridge, UK) (33) and reads were trimmed using Trim Galore (Babraham Bioinformatics, Cambridge, UK) (34). Reads were mapped to the reference human genome (Ensembl Homo_sapiens. GRCh38.80 GTF file, Cambridge, UK;

Figure 1. (A) KT cells (human bronchial epithelial cells immortalized with hTERT and CDK4) were sequentially transduced with lentiviral constructs to create a series of cell lines with different genotypes. (B) A mean of 49% of cells were positive for sex-determining region Y-box 2 (SOX2) on induction with doxycycline. (C) Western blot with a range of the cell lines used with different genotypes. Scale bars = 100 μ m. The expression of SOX2 was tightly controlled and only induced in the presence of M2 reverse tetracycline transactivator (M2rtTA) and doxycycline. Cells were positive for keratin 5 and p63 in the presence and absence of doxycycline. CDK4 = cyclin-dependent kinase 4; DAPI = 4',6-diamidino-2-phenylindole; Dox = doxycycline; hTERT = human telomerase reverse transcriptase; iSOX2 = inducible SOX2; KRT5 = keratin 5; p53lo = TP53 low; shp53 = short hairpin RNA against TP53; TP53 = tumor promoter 53.

www.ensembl.org/Homo_sapiens/Info/Index) using STAR (Cold Spring Harbor, NY) (35). The number of reads that map to a genomic feature was calculated using HTSeq (Heidelberg, Germany; <http://www-huber.embl.de/HTSeq/doc/overview.html>) (36) and differential expression analysis was performed using the counted reads and the R package edgeR (v3.8.6; Bioconductor, Seattle, WA) (37). We used the classic edgeR approach to make pairwise comparisons between treated and control groups. This

involves computing an exact test for each gene and correcting for multiple testing. EdgeR reported a *P* value for differential expression and a false discovery rate adjusted for multiple testing. Pathway analysis was undertaken using Ingenuity Pathway Analysis (Qiagen USA, Valencia, CA) restricted to significantly altered genes at a *P* value of less than 1×10^{-6} . An expression heat map was produced using Multiple Experiment Viewer (Rockville, MD; <http://mev.tm4.org/#/> welcome) and plotted by scaling the

normalized counts per million of each gene across biological replicates (38). Sequencing reads are deposited in a public database hosted by the National Center for Biotechnology Information (Sequence Read Archive Bioproject No. PRJNA344067; Bethesda, MD).

Statistical Analysis

Data were analyzed using GraphPad Prism software (GraphPad Software Inc., San Diego, CA) using standard settings. Details of specific analyses are provided in the figure legends.

Figure 2. (A) Human bronchial epithelial cells immortalized with human telomerase reverse transcriptase and cyclin-dependent kinase 4, known as KT cells, were established in short-term culture on top of a disc consisting of Type I collagen and pulmonary fibroblasts. (B) Supplementation of medium in the lower chamber with doxycycline induces the expression of red fluorescence in A549 cells transduced with an inducible red fluorescent protein reporter gene that are in established confluent culture at the air-liquid interface (ALI). Scale bars = 400 μ m. Transduced human bronchial epithelial cells were established in the organotypic system described in A; (C) doxycycline supplementation in the lower chamber led to induction of sex-determining region Y-box 2 (SOX2) expression in the monolayer. (D–H) Genetically manipulated KT inducible SOX2 (iSOX2) and tumor promoter 53 knockdown (KTiSOX2p53lo) cells retain the capacity to differentiate into constituent cells of the human airway epithelium. Scale bars = 50 μ m. ALI Transwell cultures using differentiation medium at Day 23 show a mixed population of cells with clear differentiation into ciliated cells (D and E) with acetylated tubulin representing mature cilia in red; (F) mucin-secreting (goblet) cells (MUC5AC, green; γ -tubulin/cilia basal bodies, red); (G) Club cells (CC10, green; acetylated tubulin, red); and (H) basal cells (KRT5, green; acetylated tubulin, red). (E–H) Scale bars = 50 μ m. dox = doxycycline; CC10 = club cell 10; KRT5 = keratin 5; MUC5AC = mucin 5AC; TUB = tubulin.

Figure 3. (A) A cell population growth assay was performed on KT cells (human bronchial epithelial cells immortalized with human telomerase reverse transcriptase and cyclin-dependent kinase 4) in the presence and absence of doxycycline. Induction of sex-determining region Y-box 2 (SOX2) expression did not lead to an increase in the number of cells over 120 hours. Induction of SOX2 expression did not alter cell cycle distribution in standard culture conditions but led to a significant increase in the proportion of cells in S-G2/M in the organotypic culture (OTC; *B*, paired Student's *t* test, two tailed) and in total epithelial cell number

Results

HBEC-KT cells were sequentially transduced with lentiviral constructs to create a series of cell lines with multiple genotypes including tetracycline response element–SOX2 (inducible SOX2 [iSOX2]) (Figure 1A). SOX2 expression was readily induced in the presence of doxycycline in those cell lines that expressed both SOX2 and the M2 reverse tetracycline transactivator (M2rtTA). Up-regulation of SOX2 was apparent after 24 hours in standard tissue culture. Further genotypes included a stable knockdown of TP53 alone (p53lo) and a compound genotype with iSOX2 and TP53 knockdown (iSOX2p53lo). The expression of the key markers associated with squamous differentiation—K5 and p63—was retained after SOX2 induction with doxycycline in cells with iSOX2 alone and iSOX2/p53lo.

We established a 3D OTC system in which SOX2 expression could be induced after cells were established in a monolayer at the ALI (Figure 2A). Using A549 cells transduced with a lentiviral reporter vector, we demonstrated that the addition of doxycycline to the medium in the lower chamber was sufficient to induce expression of a red fluorescent reporter in the bronchial epithelial monolayer (Figure 2B). The successful induction of SOX2 in KTiSOX2/KTiSOXp53lo cells cultured at the ALI in the OTC was demonstrated (Figure 2C). We then demonstrated that the genetically manipulated KTiSOX2p53lo cells retain the previously reported potential of the parental cells (39) to differentiate into the constituent cells of the adult human airway when cultured at the ALI on collagen-coated Transwell inserts and using bronchial differentiation medium. The KTiSOX2p53lo cells differentiated into ciliated cells, mucin-secreting cells, and Club cells, as well as basal cells (Figures 2D–2H). Therefore, despite multiple rounds of genetic manipulation, these cells retain the capacity for “normal” differentiation.

The response to SOX2 induction was compared in standard cell population assay

conditions and the OTC system using cell cycle analysis. SOX2 induction in standard conditions did not lead to an increase in cell numbers (Figure 3A), and there was no alteration in the cell cycle profile (Figure 3B). However, in the organotypic system, induction of SOX2 in the confluent bronchial epithelial monolayer led to a significant increase in cells in S/G2/M (Figure 3B) and in total cell number (Figure 3C).

Consistent with the cell cycle results, acute deregulation of SOX2 expression led to an early phenotypic difference in the organotypic model; using phase-contrast microscopy, as early as 72 hours after the induction of SOX2 expression there were focal “outgrowths” in the confluent monolayer, with cells appearing to grow on top of each other. Loss of p53 was also sufficient for modest focal outgrowths to appear in the monolayer. Stable transduction with the equivalent empty lentivirus had no phenotypic impact (see Figure E1 in the online supplement). The combination of SOX2 deregulation and p53 knockdown led to a much more dramatic phenotype, with diffuse outgrowths (Figures 3D–3I).

Histological analysis corroborated these findings (Figures 3J–3O). The “outgrowths” were areas in which there was increased thickness of the epithelial layer with apparent loss of contact inhibition and cells protruding into the putative bronchial lumen. The cells also had a high nuclear-to-cytoplasmic ratio. These outgrowths were focal for the KTiSOX2 genotype and more diffuse for the iSOX2p53lo genotype. They were consistent with focal areas of dysplasia arising from an epithelial monolayer.

We performed immunofluorescence and immunohistochemistry, and demonstrated that the dysplastic foci in doxycycline-treated KTiSOX2 and KTiSOX2p53lo cultures were positive for SOX2, and that there were no SOX2-positive cells in the control (untreated) cultures (Figures 4A and 4B, Figure E2).

We next assessed the impact of deregulated SOX2 expression on key downstream signaling pathways. In SQC,

there is typically up-regulation of phosphoAKT (pAKT) and down-regulation of pERK—signaling features that distinguish it from adenocarcinoma (6). SOX2 expression was associated with up-regulated pAKT in dysplastic foci and a marked reduction in intensity of cytoplasmic pERK staining, recapitulating the signaling patterns reported in more advanced human SQC (Figures 4C–4J).

We compared the findings in this organotypic model with a series of high-grade preinvasive lesions from a tissue microarray. Lesions from 15 patients were assessed, 9 of which were from patients with simultaneous invasive SQC. SOX2 is expressed in normal human bronchial epithelium and in basal cell hyperplasia, but, at steady state, Ki67-positive cells are relatively rare and tend to be adjacent to the basement membrane (Figures 5A and 5B, Figure E3). SOX2 was expressed in high-grade dysplastic lesions from 14 of 15 patients, qualitatively at a higher level than that seen in normal bronchial epithelium or basal cell hyperplasia (Figures 5C and 5E, Figure E3), and it was associated with an increased proliferative index (Figures 5D and 5F). Unfortunately, staining for pAKT was unreliable in these archived specimens that had undergone variable processing. However, in one lesion in which a clear transition from normal epithelium to high-grade dysplasia was captured, pAKT expression was restricted to a few cells abutting the basement membrane in the pseudostratified epithelium, whereas, in the dysplastic lesion, there was significant up-regulation of SOX2, Ki67, and pAKT throughout the epithelium (Figures 5G–5I).

SOX2 is a pleiotropic nuclear transcription factor that exerts variable phenotypic effects in different cell types and cell culture systems (40–42). Deregulated SOX2 has been shown to directly regulate cyclinD1 and B-cell lymphoma 2 (BCL2) in breast cancer and melanoma, respectively (40, 41). We performed ChIP-PCR on KTiSOX2p53lo cells at the ALI in the presence and absence of doxycycline. We demonstrated that cyclinD1 and BCL2 promoters were specifically bound by SOX2

Figure 3. (Continued). (C, paired Student's *t* test, two tailed). Data presented in A represents means ($\pm$ SD) of three separate experiments in triplicate. (D and E) Control cell lines (KT-M2 reverse tetracycline transactivator [M2rtTA] [KTM2]) were treated with doxycycline with no significant alteration in morphology on phase-contrast or histology. (H) Loss of p53 led to modest focal outgrowths in the monolayer. Induction of SOX2 (inducible SOX2 [iSOX2]) led to an obvious phenotype with focal (KTiSOX2; F and G) and more diffuse (KTiSOX2 tumor promoter 53 [TP53] knockdown [KTiSOX2p53lo]) changes (H and I) in the epithelial monolayer. (J–O) Histological analysis was consistent with the changes seen using phase-contrast microscopy. There were diffuse outgrowths demonstrated in the KTiSOX2p53lo upon induction of SOX2 expression in the confluent monolayer. The histological features were typical of high-grade preinvasive lesions: loss of contact inhibition, a thickened epithelial layer with dysplasia, absence of maturation, and a high nuclear-to-cytoplasmic ratio. (D–F) Scale bars = 400 μ m; (G–I) scale bars = 1,000 μ m; (J–O) scale bars = 100 μ m. Dox = doxycycline; NS = not significant. **P* $\leq$ 0.05.

Figure 4. Activation of sex-determining region Y-box 2 (SOX2) leads to focal dysplastic changes and alterations in key cell signaling cascades. (A and B) Dysplastic lesions in doxycycline-treated cultures were strongly positive for SOX2 with no SOX2 staining in the untreated cultures. (C–J) Serial sections from dysplastic lesions show a down-regulation in phospho-extracellular signal-regulated kinase (pERK) and an up-regulation in phosphoAKT (pAKT) in those cells that are SOX2 positive and in cycle (Ki67 positive). Scale bars = 100 μ m. DAPI = 4',6-diamidino-2-phenylindole; dox = doxycycline; H+E = hematoxylin and eosin stain; Ki67 = marker of proliferation Ki67; KT = human bronchial epithelial cells immortalized with human telomerase reverse transcriptase and cyclin-dependent kinase 4; KTiSOX2p53lo = KT inducible SOX2 and tumor promoter 53 (TP53) knockdown.

(Figure 6A), and that both target oncogenes were up-regulated (Figures 6B and 6C).

A broader survey of key cell cycle regulatory proteins showed that many were up-regulated (Figure 6C), and that cyclin-dependent kinase inhibitor 1 (CDKN1A), a key negative regulator of the cell cycle (p21), was down-regulated.

The results are consistent with SOX2 acting as a mitogenic oncogene and targeting, in this novel organotypic system, key downstream effectors anticipated from other experimental systems. SOX2 is a pleiotropic nuclear transcription factor that controls the expression of large gene sets that vary depending on the cell type and experimental system (43, 44). We therefore undertook RNA-Seq analysis to perform an unbiased transcriptomic analysis of the broader impact and potential downstream effectors of deregulated SOX2 in this OTC model of bronchial dysplasia. Over 2,000 genes were significantly deregulated by SOX2 induction ($P < 0.01$), and there was clear segregation between the SOX2-induced and noninduced experiments (Figure E4, Sequence Read Archive Bioproject No. PRJNA344067). Further analysis was undertaken using ingenuity pathway analysis with a cut-off of significance of a P value less than 1×10^{-6} (Qiagen). Importantly, and consistent with the observed phenotype in the OTC system, the most significantly altered cellular functions associated with deregulated SOX2 expression were “cellular movement” and “cellular growth and proliferation” (Figure 7A). The 197 genes annotated as “cell migration” show clear segregation between SOX2-deregulated and control experiments, and include many genes previously implicated in carcinogenesis and known direct targets of SOX2, such as ETS translocation variant 4 (ETV4) and dickkopf-related protein 1 (DKK1) (45, 46) (Figure 7B and Table E1).

EMT is a hallmark of cancer implicated in the critical early stages of the disease, including cell migration. We used qPCR to corroborate RNA-Seq results and analyze an extended panel of canonical mediators of EMT (Figure 7C). The RNA-Seq data also implicated SOX2 in the up-regulation of emerging drivers of EMT and cell migration phenotypes, including anterior gradient protein 2 homolog (AGR2), cell migration-inducing hyaluronan-binding protein (CEMP), and serpin family I member 1 (SERPINI1) (47–49), also confirmed using qPCR (Figure 7D). Of these, SERPINI1 is most

Figure 5. Representative images from a tissue microarray with high-grade preinvasive bronchial lesions. (A and B) Basal cell hyperplasia has weakly positive staining for sex-determining region Y-box 2 (SOX2) and rare Ki67 (marker of proliferation Ki67)-positive cells. (C–F) High-grade dysplastic lesions exhibit much more marked SOX2 and Ki67 staining that extends throughout the epithelial layer. PhosphoAKT (pAKT) staining proved challenging on archived tissue microarray specimens; however, (G–I) there was a single sample in which the border between normal, ciliated, pseudostratified epithelium, and high-grade dysplasia was available that provided a robust internal control for the antibody performance. There was a clear up-regulation in pAKT staining in those dysplastic cells in which SOX2 and Ki67 were up-regulated. Scale bars = 100 μm.

Figure 6. Deregulated sex-determining region Y-box 2 (SOX2) targets key cancer-related genes in the organotypic culture system. Chromatin immunoprecipitation–polymerase chain reaction (PCR) was performed using input cross-linked DNA and the same DNA incubated with both a SOX2-specific antibody and an IgG control. There was enrichment for the B-cell lymphoma 2 (*BCL2*) and cyclinD1 (*CCND1*) promoters in the SOX2 pulldown treated with doxycycline (Dox), but not the untreated (No Dox) nor the IgG control, confirming that SOX2 binds both gene promoters and regulates their transcription in this system (A). (B and C) Quantitative PCR (qPCR) experiments confirmed that *BCL2* (B) and *CCND1* (C) were both up-regulated transcriptionally on induction of SOX2. The expression of a series of cell cycle genes was analyzed. (C) There was up-regulation of key cyclin-dependent kinases (*CCNA2*, *CCNB1*, *CCNB2*, and *CCNE2*), and down-regulation of cyclin-dependent kinase inhibitor 1 (*CDKN1A* or p21), a critical inhibitor of cell cycle progression. These alterations were seen on treatment with doxycycline compared with the untreated control. The following kinases were also tested but were not significantly altered: *CCNA1*, *CCNE1*, *CCND2*, and *CCND3*. The results shown are representative of three independent experiments, and data are presented as the mean (n = 3) (±SD). For the qPCR experiments, $\Delta\Delta$ threshold cycle values were log (base 10) transformed, and results for each marker were compared with glyceraldehyde phosphate dehydrogenase. Statistical analysis: a Student's *t* test (unpaired) was performed to test significance: **P* ≤ 0.05; ***P* ≤ 0.01; ****P* ≤ 0.001; and *****P* ≤ 0.0001. Ab = antibody.

frequently altered in early lung squamous cell carcinoma, often in conjunction with SOX2 (Figure 7E). We confirmed that SERPINI1 up-regulation was accompanied by protein overexpression (Figure 7F). We then optimized primer sets around the SERPINI1 transcriptional start site to demonstrate that it was a direct transcriptional target of SOX2 (Figure 7G). We then corroborated this with clinical samples and demonstrated that SERPINI1 is expressed in high-grade SOX2-positive bronchial dysplastic lesions, but not in basal cell hyperplasia (Figure 7H). We have previously demonstrated that TP53 mutated, SOX2-amplified clonal populations expand, migrate, and colonize the respiratory epithelium. The current

data comprehensively link SOX2 mechanistically to this clinically important preinvasive migratory phenotype (50).

Finally, building on the immunohistochemistry (Figure 5) and the published literature linking activation of the phosphatidylinositol-4,5-bisphosphate 3-kinase (PI3K)–AKT pathway and bronchial preneoplasia (51–54), we assessed the impact of a specific inhibitor of protein kinase-B (AKT) in preventing the development of SOX2-driven bronchial dysplasia in this organotypic model system. AZD5363 is a potent and specific inhibitor of AKT1–3 that is already in clinical trials (55). AZD5363 had a clear impact on reducing the dysplastic phenotype (Figures 8A–8D). No dysplastic

outgrowths were seen on phase-contrast microscopy in the treated cultures, and this was confirmed by histology. However, the epithelial cells in contact with the collagen basement membrane and at the ALI were apparently healthy, entering cell cycle and expressing SOX2 (Figures 8E and 8F), indicating that there was not general severe cellular toxicity as a result of the AZD5363 treatment.

Discussion

In this work we provide direct evidence that deregulation of a putative oncogene, SOX2, in the appropriate molecular and cellular context, and with the appropriate microenvironmental cues (collagen, fibroblasts, ALI), is sufficient to drive bronchial dysplasia in a short-term 3D culture system.

The cellular, molecular, and environmental context in which an oncogene becomes deregulated is critical to its impact. The cells used in this study are immortalized HBECs that have been well characterized. They have an almost normal karyotype, intact p53 signaling, and express markers, K5 and p63, that are associated with airway basal cells—the putative cells of origin of SQC (27). They have previously been used in experiments examining the role of mutated V-Ki-ras2 Kirsten rat sarcoma viral oncogene homolog (KRAS) with or without the expression of v-myc avian myelocytomatosis viral oncogene homolog (MYC) in carcinogenesis (11, 56). KRAS mutations are very common in lung adenocarcinoma, but very rare in SQC. In this work, we model the key events reported in bronchial dysplasia/early squamous carcinogenesis.

When cultured using specific medium and at the ALI, the HBECs can differentiate into the constituent cells of the bronchial epithelium, including secretory (Club) and ciliated cells (39). If cultured in matrigel, they have the capacity to form branching structures and express differentiation markers consistent with distal airway differentiation (39). In this study, we show that, notwithstanding multiple rounds of defined genetic manipulation to create the complex genotype that we sought, they retain their capacity to differentiate into constituent airway cell types. In contrast, a commonly used SV40 large T antigen immortalized bronchial epithelial cell line, BEAS2B, reportedly does not differentiate at the ALI (57).

The limited available molecular epidemiological data from human samples

Figure 7. The most significantly differentially expressed genes ($P < 1 \times 10^{-6}$, $n = 832$) were analyzed using ingenuity pathway analysis. (A) The most significantly altered molecular and cellular functions were cellular movement and cellular proliferation. (B) A heatmap of 197 genes associated with cell migration illustrating up- and down-regulation of genes with induction of sex-determining region Y-box 2 (SOX2) is presented. The results show three paired replicates with (*italics*) and without (*nonitalic font*) SOX2 induction. A number of genes are highlighted, including reported direct targets of SOX2 (ETS translocation variant 4 [*ETV4*] and

Figure 8. Representative phase-contrast (A and B) and hematoxylin and eosin stains (C and D) of doxycycline-treated (A and C) and doxycycline (Dox) and AZD5363-treated (B and D) organotypic cultures. All test and control cultures were treated with dimethyl sulfoxide. As before, induction of sex-determining region Y-box 2 (SOX2) led to a diffuse dysplastic phenotype. (B and D) Treatment with a pan-AKT inhibitor (AZD5363, 10 μ M) abrogated the development of the dysplastic lesions. Cells in the treated culture were alive and continued to express SOX2 (E) and Ki67 (F). Images are representative of three independent experiments. (A and B) Scale bars = 1,000 μ m; (C–F) scale bars = 100 μ m. KT = human bronchial epithelial cells immortalized with human telomerase reverse transcriptase and cyclin-dependent kinase 4; KTISOX2 p53lo = KT inducible SOX2 and tumor promoter 53 (TP53) knockdown.

suggest that p53 is disrupted early in the pathogenesis of SQC and that 3q amplification defines the transition between low- and high-grade dysplasia (12, 16, 58, 59). The HBECs in this study are immortalized by the introduction of two specific genes, CDK4 and hTERT. CDK4 overexpression overcomes the cell cycle inhibition mediated by the p16INK4A-RB tumor suppressor pathway, and hTERT (telomerase) facilitates clonal expansion through crisis (60). There are limited data on CDKN2A (p16INK4A locus at 9p21) in preinvasive disease, but Wistuba and colleagues (59) reported loss of heterozygosity at 9p21 in dysplasia and carcinoma *in situ*, and the CDKN2A locus is disrupted in up to 75% of SQCs (14). Telomerase overexpression increases with dysplastic progression in

immunohistochemical studies (61). Therefore, the deregulated overexpression of SOX2 in the current model is in a rational cellular and molecular context that reflects the human disease.

Many groups have previously demonstrated the importance of the physical and cellular microenvironment in the phenotypic response to oncogenic stimuli. The data we present are consistent with these studies and emphasize the significant benefit of using organotypic, as opposed to traditional, cell culture. The system adopted here reflects that used by others studying the pathobiology of bronchial epithelial cells. We show that it is possible to genetically manipulate the epithelial cells and induce deregulated expression of SOX2 when an intact respiratory

epithelial monolayer at the ALI is already established.

SOX2 has previously been implicated as an oncogene in the pathogenesis of SQC. In a mouse model of SQC, almost 50% of mice develop cancer 9 months after inhalation of a virus that contains SOX2 and deletes liver kinase B1 or serine/threonine kinase 11. The majority of the cancers that developed were of the squamous subtype (6). SOX2 was also sufficient to transform SV40 large T antigen-transformed BEAS2B cells (20), but was insufficient to transform similarly immortalized small airway epithelial cells alone (19). SV40 large T antigen has multiple impacts on key tumor suppressor pathways, including p53 and RB, and these cell lines have not been characterized karyotypically. Therefore, it is difficult to measure the impact of SOX2 in these cellular contexts.

Here, we provide novel data regarding the impact of SOX2 in an organotypic human system with a well characterized cell line that has been nonvirally immortalized and has an almost normal karyotype. The data demonstrate that SOX2, in the appropriate molecular and physical environmental context, drives dysplastic change. Moreover, its impact when deregulated is relatively acute—outgrowths from the monolayer are readily visible at 72 hours. Furthermore, the combination of SOX2 deregulation and p53 knockdown leads to an impressive dysplastic phenotype. This is entirely consistent with what has been previously demonstrated in studies on human specimens: p53 loss/disruption is likely to be a very early event in the pathogenesis of this disease, and SOX2 deregulation is superimposed on it at the key epidemiological and molecular bottlenecks between low- and high-grade dysplasia (12, 13, 16). We observed in this model similar signaling alterations to those reported in the invasive disease and previously reported in preinvasive disease (52, 53). We then used a unique tissue

Figure 7. (Continued). dickkopf-related protein 1 [*DKK1*] and those directly implicated in epithelial-mesenchymal transition (EMT; *CDH2*, *CEMIP*). Further details are in Table E1. (C) Quantitative polymerase chain reaction (qPCR) of canonical mediators of EMT was performed to corroborate RNA-Seq results and confirmed their up-regulation by SOX2. In addition, a number of the most significantly altered genes in differential gene expression analyses (*CEMIP*, *SERPIN1*, and *AGR2*; all $P < 1 \times 10^{-13}$) were recently implicated in EMT in the literature but not annotated in the “cellular movement” group. (D) qPCR experiments confirmed significant up-regulation in each case. (E) Interrogation of The Cancer Genome Atlas database showed that *SERPIN1* was the most commonly altered of these in early squamous lung cancer and commonly altered with SOX2. The corresponding protein, SERPIN1, was also overexpressed on SOX2 induction in the organotypic culture (F), and chromatin immunoprecipitation-PCR confirmed enrichment for SOX2 binding near the *SERPIN1* transcriptional start site (G). (H) Further sections from the tissue microarray were stained for SERPIN1 and showed clear cytoplasmic staining in biopsies of high-grade dysplasia (upper panels) compared with normal epithelium. This is particularly reinforced by the section containing the transition from relatively normal pseudostratified epithelium to disorganized high-grade dysplasia (lower panel). * $P \leq 0.05$, ** $P \leq 0.01$, *** $P \leq 0.001$, and **** $P < 0.0001$. Ab = antibody; *AGR2* = anterior gradient protein 2 homolog; *CDH2* = cadherin 2; *CEMIP* = cell migration inducing hyaluronan binding protein; CPM = counts per million; Dox = doxycycline; *LEF1* = lymphoid enhancer binding factor 1; *MMP10* = matrix metalloproteinase 10; ns = not significant; *SERPIN1* = serpin family I member 1; *SNAI* = snail family transcriptional repressor; *TWIST* = twist family basic helix-loop-helix transcription factor 1.

microarray of human specimens to demonstrate that the increased proliferative index seen in high-grade dysplastic lesion coexists with an increase in SOX2 expression.

SOX2 is a pleiotropic nuclear transcription factor that exerts variable phenotypic effects in different cell types and cell culture systems. In driving dysplastic change in this model system, we show that it directly regulates the expression of key proteins implicated in carcinogenesis including cyclinD1, required for progression through G1; and BCL2, a negative regulator of apoptosis. We further show that overexpression of SOX2 up-regulates multiple kinases involved in cell cycle progression and down-regulates p21, a potent cyclin-dependent kinase inhibitor. Taken together with the presented Ki67 and cell cycle data, this shows that SOX2 is driving cell proliferation, consistent with the results seen in the tissue microarray staining of human specimens. Furthermore, we demonstrated that the phenotypic changes associated with deregulated SOX2 are accompanied by a broad transcriptional program encompassing multiple hallmarks of cancer—cell proliferation, inhibition of apoptosis, and cell migration—including canonical and emerging genes associated with EMT and metastasis. Serine protease inhibitors have diverse functional roles, but recent data strongly associate SERPINI1 with EMT and lung cancer metastatic cerebral invasion (62). This is the first study to show that SOX2 is a direct transcriptional regulator of *SERPINI1* and to demonstrate that it is overexpressed at a preinvasive stage in clinical specimens. SOX2 orchestrates a complex genetic program resulting in the dysplastic phenotype. Although nuclear transcription factors are notoriously challenging drug targets, emergent medicinal chemistry strategies, and these data, strongly support the direct therapeutic targeting of SOX2.

EMT is regarded as a key early event in the pathogenesis of epithelial cancers. We and others have shown previously that cell

migration is also a key early feature of SQC, and that SOX2-amplified/p53-disrupted clonal populations *in vivo*, as reproduced in the OTC, have the capacity to migrate and colonize the bronchial epithelium (12, 13). Taken together, these data suggest that SOX2-driven cell migration is a mechanism underpinning the so-called field-change effect.

No model system can recapitulate the *in vivo* disease perfectly. In previous work, we have shown that the amplification of SOX2 is likely to be an incremental process (13), whereas, in this case, SOX2 is turned on over a very short time period. It is not possible to correlate the SOX2 expression levels in the OTC to human preinvasive specimens. However, we have shown that SOX2 is amplified and significantly overexpressed in preinvasive lesions (16). Furthermore, there are clear data from the TCGA (The Cancer Genome Atlas) studies that show that the expression of SOX2 is grossly elevated in SQC compared with other tumor types (14) (Figure E5). The *in vivo* complexity, in terms of immunological response and the microenvironment, cannot yet be recapitulated *in vitro*. Finally, doxycycline binds collagen, and data using a fluorescent reporter suggests that it continues to be present at levels that maintain transgene expression for at least 3 weeks after withdrawal from the medium (data not shown). Therefore, withdrawal experiments to establish the need for continued SOX2 expression in the maintenance of these outgrowths were not possible.

The confirmation of the impact of SOX2 overexpression as an early driver of bronchial carcinogenesis and the ability to phenocopy the human disease in short-term culture are important steps forward that should help to meet a key challenge—the development of agents to use for primary and secondary chemoprevention (63). We have begun to address this challenge—we demonstrated that AZD5363, a targeted inhibitor of AKT already in early-phase clinical trials,

completely abrogated the development of the dysplastic phenotype. This is consistent with prior studies suggesting that the PI3K–AKT axis may be an appropriate target for intervention in bronchial preneoplasia, including a landmark clinical trial that showed that this pathway could be targeted in a chemoprevention strategy (51–54). The current study mimics using AZD5363 to prevent the emergence of bronchial dysplasia. It has a side-effect profile that would be likely to preclude its use in primary chemoprevention of at-risk groups, but there may be patients in whom a trial of this or a similar compound may be appropriate in the secondary chemoprevention setting.

This platform will be suitable for adaptation to moderate throughput screening of potential chemoprevention compounds, and further screens for potential complementary genetic lesions that may drive the progression of this devastating disease. ■

Author disclosures are available with the text of this article at www.atsjournals.org.

Acknowledgment: The authors thank Professor J. W. Shay and Professor J. D. Minna, University of Texas (Dallas, TX) for the kind gift of the human bronchial epithelial cell-3 KT cells, Dr. Luca Pellegrinet (University of Cambridge, Cambridge, UK) for assistance with flow cytometry, Nicholas Salisbury (University of Cambridge, Cambridge, UK) for A549 pTRIPZ-RFP cells, and the histology core at the Wellcome Trust Medical Research Council Cambridge Stem Cell Institute for histological services (Cambridge, UK). FUW tetO-lox-hSOX2 and FUW M2 reverse tetracycline transactivator were gifts from Rudolf Jaenisch (Massachusetts Institute of Technology, Boston, MA) (Addgene plasmid nos. 20,324 and 20,729), and pLVU-hshp53-tTR-KRAB and pLVU-tTR-KRAB were gifts from Patrick Aebischer and Didier Trono (Ecole Polytechnique Federale de Lausanne, Lausanne, Switzerland) (Addgene nos. 11,645 and 11,654). The authors also thank Cambridge Genomic Services (Cambridge, UK) for able assistance with RNA-Seq and bioinformatic analyses.

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