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Antitumour efficacy of the selumetinib and trametinib MEK inhibitors in a combined human airway–tumour–stroma lung cancer model

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ABSTRACT

With more than 1 million deaths worldwide every year, lung cancer remains an area of unmet need. Accessible human *in vitro* 3D tissue models are required to improve preclinical predictivity. OncoCilAir™ is a new *in vitro* model of Non Small Cell Lung Cancer which combines a reconstituted human airway epithelium, human lung fibroblasts and lung adenocarcinoma cell lines. Remarkably, we found that in this 3D microenvironment tumour cells expand by forming nodules, mimicking a human lung cancer feature. OncoCilAir™ mutated for KRAS and expressing the green fluorescent protein were used to test the antitumour potential of the investigational MEK inhibitors selumetinib and trametinib. As primary endpoint, changes in tumour size were assessed by fluorescence measurements. Tumours showed a reduced growth in response to the MEK inhibitors, but halting the selumetinib dosing resulted in tumour relapse. Importantly, toxicity study on the normal part of the cultures revealed that the airway epithelium integrity was also affected by anticancer drug treatments. These results highlight the possibility to assess simultaneously drug efficacy, drug side-effect and tumour recurrence within a single culture model. OncoCilAir™ heralds a new generation of integrated *in vitro* tumour models that should be valuable tools for drug development, while reducing animal testing.

1. Introduction

Lung cancer remains a major public health problem with an estimated 1.6 million new cases diagnosed worldwide each year (Malvezzi et al., 2013). To date there is no effective treatment for patients (Hay et al., 2011) and unfortunately, a large number of promising drug leads continue to fail in late clinical stages (Arrowsmith and Miller, 2013). This observation has casted uncertainty on the established drug discovery process and doubt about the relevance and the predictive validity of the pre-clinical animal models currently in use (Scannell et al., 2012). Fundamental differences between the animal and human biology, notably regarding transcriptional and telomerase regulation (Forsyth et al., 2002; Odom et al., 2007), cytokine and matrix metalloproteinases production (Dorman et al., 2010; Seok et al., 2013), compromise the validity of the animal experiments (Mak et al., 2014; Rangarajan and Weinberg, 2003). Even the most advanced class of *in vivo* preclinical model, namely the patient derived xenograft model, displays limitations due to the rapid replacement of the original human stroma environment by murine components (Julien et al., 2012). Such an inappropriate environment changes tumour growth properties and ultimately influences therapeutic responses (Lee et al., 2014; Straussman et al., 2012). Consequently, the subtle crosstalk taking place between the tumour bulk and its microenvironment has become a key consideration for the development of anticancer drugs (McMillin et al., 2013). Indeed, solid tumours are able to hijack properties of mesenchymal cells, like fibroblasts, to promote their own growth and progression. Moreover, they are also dependent on both mechanical and molecular signals coming from the adjacent healthy environment (Chen et al., 2014; Egeblad et al., 2010). Thus, tumour formation is the result of a continuous co-evolution of cancer cells and their microenvironment (Junttila and de Sauvage, 2013). Many strategies to integrate this complexity in three-dimensional (3D) *in vitro* models, such as organotypic cultures (Vaira et al., 2010), decellularized lung matrix (Mishra et al., 2012), bioscaffold (Godugu et al., 2013) and spheroids (Amann et al., 2014) have been explored. However, they all have significant limitations (short term growth, limited production, no stroma,

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and no lung environment) and only partially reproduce the *in-vivo* situation of the disease (Lovitt et al., 2014). In that context, the development of an accessible human *in vitro* 3D tumour tissue model recapitulating lung cancer would be highly valuable. Here we report the bioengineering of a new generation of lung cancer models, OncoCilAir™, which combine a functional airway epithelium, primary lung fibroblasts and developing tumour nodules. In this study, a KRAS mutated Non Small Cell Lung Cancer (NSCLC) cell line was used to build the OncoCilAir™ tumour component. We first confirmed through histological and functional characterisation that this model closely mimics biologically relevant parameters of the *in vivo* situation. Then, we assessed its relevance as a predictive tool for anticancer drug evaluation. To that purpose, we performed an efficacy study using selumetinib and trametinib, two competing MEK inhibitors currently involved in KRAS-mutant lung cancer clinical trials (Janne et al., 2013; Wright and McCormack, 2013), and the standard anticancer agent docetaxel. The EGFR-TK inhibitor erlotinib was included as a negative control. We found a reduced growth of tumours in response to the MEK inhibitors confirming OncoCilAir™ as a *bona fide in vitro* lung cancer model.

2. Material and methods

2.1. Development of in vitro 3D lung tumour model OncoCilAir™

OncoCilAir™ is a complex cellular model based on the co-culture of three different human components: bronchial cells, lung fibroblasts and NSCLC cell lines. The resulting tissue consists in a functional respiratory epithelium which comprises developing tumour nodules. Lung fibroblasts were derived from small bronchi explants cultured in DMEM supplemented with 10% fetal calf serum, seeded onto inverted 33 mm^2 Transwell inserts (Ref Costar #3470, Cambridge, MA) and allowed to attach for 3 h at 37 °C. The Transwell inserts were then turned back and transferred to 24-well plates. The respiratory epithelium part was reconstituted from primary human airway epithelial cells (hAECs) isolated from patient's bronchial biopsies by enzymatic digestion and grown in a commercially available defined airway culture medium (EP04MM from Epithelix, Switzerland). Briefly, 2.5×10^5 hAECs were plated on top of the porous membrane undercoated with fibroblasts. NSCLC cell lines, such as A549, were tagged with a lentivirus expressing the green fluorescent protein (GFP) and incorporated in each insert of the 24-well plates at different cell concentrations (0.05–1 million cells per well, depending on cell lines) to obtain the optimal seeding density required for the formation of tumours nodules. Two days after seeding, hAECs were switched to an air–liquid interface (ALI) for at least 25 days to obtain differentiation into a ciliated pseudostratified airway epithelium. All human biopsies were obtained according to the local ethical committee requirements. Experimental procedures were explained to patients and all of them signed an informed consent.

2.2. Histology and immunocytochemistry

Tissues cultures, 30 days *in vitro*, were rinsed in PBS and fixed by immersion in 4% formaldehyde for 30 min. Fixed tissues were embedded into paraffin, sectioned at 5 μm and processed for staining with Hematoxilin Eosin (H&E) and Alcian Blue before analysis by light microscopy. Immunocytochemistry on paraffin-embedded tissues with monoclonal mouse anti-human p63 protein (dilution 1:100, 4A4 clone, #M7247, Dako) or monoclonal mouse anti-human Ki-67 antigen (dilution 1:150, MIB-1 clone, #M7240, Dako) was followed by biotinylated secondary antibodies and diaminobenzidine detection according to manufacturer's instruction (Envision™ kit, Dako). Cell nuclei were counterstained using Mayer Hemalun coloration. Digital images of HE and immunostained slides were acquired using a Zeiss AxioCam microscope. Proliferation rate was determined by counting the ratio of Ki-67 expressing cell nuclei to the total number of cell nuclei in the field observed, and expressed as a percentage.

2.3. Trans-epithelial electrical resistance (TEER)

After addition of 200 μl of culture medium to the apical compartment of the tissue cultures, resistance was measured across cultures with an EVOMX volt-ohm-meter (World Precision Instruments UK, Stevenage) in triplicate for each time point. The TEER values (Ω) were converted normalised by using the following formula: $\text{TEER}\,(\Omega\,\text{cm}^2) = (\text{resistance value}\,(\Omega) - 100\,(\Omega)) \times 0.33\,(\text{cm}^2)$, where 100 Ω is the resistance of the membrane and 0.33 cm^2 is the total surface of the epithelium.

2.4. Ciliary beat frequency (CBF)

CBFs were recorded after 35 days in culture with a high speed acquisition camera. Video sequences were analysed with dedicated software (Ciliametrix) which determines CBFs by timing a given number of individual ciliary beat cycles. The CBF represents the average value of thousands of ciliated cells, expressed in Hz.

2.5. Electrophysiology in Ussing Chambers

The bioelectric properties of tissue cultures were studied at 35 days *in vitro* by placing the inserts in Ussing Chambers (Jim's Instruments, Iowa City, IA). The apical and basal chambers were filled with a Krebs buffer, maintained at 37 °C and gassed with 100% O^2. The transepithelial potential difference was voltage-clamped at 50 μA, and the resulting short-circuit current (Isc) recorded as previously described (Wiszniewski et al., 2006). Na^+ channel activity was measured by applying 100 μM amiloride to the apical solution. Isoproterenol, a β2-adrenergic receptor agonist, was added to the apical solution (10 μM) to stimulate transepithelial Cl^- through CFTR channels. Glybenclamide (100 μM) was used to block Cl^--transport.

2.6. Anticancer drugs

Selumetinib and erlotinib were purchased from Selleckchem. Trametinib was obtained from Santa Cruz Biotechnology. Docetaxel was bought from SIGMA Corporation. Drugs were diluted in DMSO, except for docetaxel (in ethanol). Drug concentrations used in the efficacy study were determined by a series of dose–response experiments in A549 monolayer culture using cell viability assay (alamarBlue, Life Technologies) after 96 h of treatment (Fig. S2). IC_{50} values were estimated by linear regression using Graphpad Prism software: 2.5 μM for selumetinib, 50 nM for trametinib, 123 μM for erlotinib (suggesting resistance of A549 cells to this drug). For docetaxel, a working concentration of 100 nM was selected based on experimental and reported data (Hernandez-Vargas et al., 2007). For erlotinib, a working concentration of 1 μM reported to be effective on lung cancer cell lines sensitive to EGFR-TK inhibitors was chosen (Yamada et al., 2013). These concentrations were then applied to OCA_KRAS^{mut} cultures in a short term experiment (3 days). As no obvious toxicity signs were observed at that time on the airway epithelium component, these concentrations were used in subsequent studies. The efficacy study was performed on two different series of OncoCilAir™, OCA562_KRAS^{mut} and OCA575 KRAS^{mut}, established with hAECs isolated from two

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