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LENA BIOSCIENCES
Booth 4709

Characterization of patient-derived organoids cultured on a gas-rich, liquid-liquid interface

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INTRODUCTION

Modeling tissues in vitro has long consisted of two-dimensional (2D) monolayer cultures grown in a dish, flask, or multi-well plate. While these methods remain adequate for some applications, the advent of personalized cancer therapies has necessitated the development of more complex models that more accurately represent the in vivo environment. Attempting to characterize tumor cell biology and response to candidate pharmaceuticals in 2D cultures has led to results that fail to translate to clinical therapies. A key source of this failure is the loss of cell diversity due to selective pressure resulting in cell populations that favor adhering to a rigid substrate. Three-dimensional (3D) cell culture methods have produced models that capture many of the dynamics of the in vivo tissue environment. Patient-derived organoids (PDOs) are quickly becoming the most attractive solution for creating predictive, patient-specific tumor models in vitro. Typically grown as spheroids, these models still present limitations in terms of cell survival and recapitulation of tumor development.

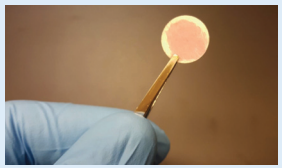
Lena Biosciences has previously developed complex, vascularized tissue models using patented 3D scaffolds and a perfusion system. Hepatocytes grown in this system demonstrated increased cell viability and recovery of cytochrome P450 enzyme activity compared to 2D controls. Integral to the perfusion system is a pure, synthetic liquid that is twice as dense as and immiscible with cell culture medium and reagents. It is permissive to atmospheric gases, providing a gas-rich, liquid-liquid interface below 3D cultures maintained in the system. We hypothesized that PDOs could be cultured without a 3D scaffold, directly at the interface between the cell culture medium and this synthetic liquid.

METHODS

Cell Culture

Cryoplateable primary human hepatocytes (BioIVT, 10-donor, mixed gender) were cultured in the manufacturer's recommended medium. HepG2 cells (ATCC® HB-8065™) were maintained in EMEM supplemented with 10% fetal bovine serum (FBS). Modified H1299 cells were a gift from Adam Marcus and were maintained in RPMI-1640 supplemented with 10% FBS. Patient-derived NSCLC cells were maintained in modified M87 medium[1]. All cells were maintained in a humidified incubator at 37 ºC.

Primary human hepatocytes were plated in 2D or seeded in Matrigel® (8 mg/mL) in SeedEZ scaffolds at a density of 333,000 cells per scaffold. Cultures were allowed to gel at 37 ºC for 10 minutes and subsequently transferred to Lena Biosciences' PerfusionPal system.



Patient-derived NSCLC cells isolation

All tissue samples were procured by Human Tissue Procurement Service of Winship Cancer Institute of Emory University. Samples were obtained from patients undergoing surgical biopsies, who gave their informed consent after approval from the IRB of Emory University following IRB protocol. Tissues were digested for 2-5 hr in digestion buffer (DMEM/F-12, 10 mM HEPES, 2% BSA, 1x ITS, 0.5 µg/ml hydrocortisone, Pen/Strep, Normocin, Fungizone) containing 2 mg/ml Type 3 Collagenase (Worthington), 100 U/ml Hyaluronidase (Sigma) at 37°C until fully digested. Cells were pelleted for 5 min at 320xg and resuspended in TAC buffer (StemCell Tech.) to remove RBCs. Cells were then re-suspended in digestion buffer containing 200 µg/ml DNase 1 (Sigma) and incubated 10 min at 37°C. Cells were then plated. Cells were grown in modified M87 media (1). Cells were deferentially trypsinized to remove fibroblasts. Absence of fibroblasts was confirmed via western blot and immunofluorescence.

CYP Activity Measurement

CYP1A1 and CYP2C19 activities were measured fluorescently using EROD and CEC assays, respectively. For the ethyxoyresorufin-O-deethylase (EROD) assay, cells were exposed to 7-ethoxyresorufin (10µM) and salicylamide (inhibitor of phase II metabolism of resorufin, 1.5mM). For the 3-cyano-7-ethoxycoumarin (CEC) assay, cells were exposed to CEC (25µM). CYP1A2, CYP2B6, CYP2C9, and CYP3A4 activities were measured using CYP-Glo assays (Promega) according to the manufacturer's instructions. Omeprazole (50µM) was used to induce CYP1A2. Rifampin (10µM) was used to induce all other CYP enzymes.

alamarBlue Assay for Metabolic Function

After 10 days *in vitro* (DIV), alamarBlue reagent was added to patient-derived NSCLC cell cultures at a volume equal to 1/10 of the volume of culture medium. The cultures were returned to the incubator for a period of 45 minutes after which a sample of the medium from each condition was transferred to a 96-well plate and read on a Biotek Synergy 4 plate reader for fluorescence (ex/em 545/590 nm).

LDH Release Assay

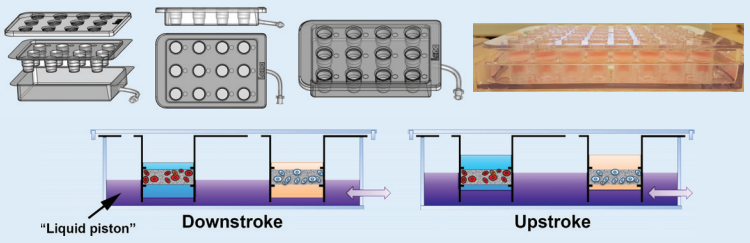
Prior to the addition of alamarBlue reagent, a sample of medium was taken to assess LDH release. Samples were centrifuged and the supernatant was transferred to fresh tubes. A samples of medium from each condition was transferred to a 96-well plate for a colorimetric LDH assay (Pierce™ LDH Cytotoxicity Assay Kit).

Statistical analysis

Statistical significance was determined by Student's t-test, One-way ANOVA and Tukey's post-hoc test, or Two-way ANOVA and Bonferroni's post-hoc test.

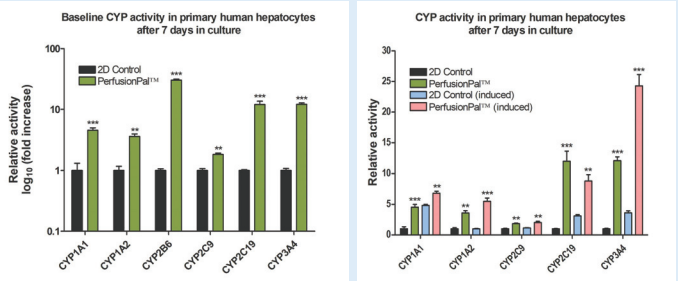
[1]DeRose, YS, et al. Patient-derived Models of Human Breast Cancer: Protocols for In vivo Applications in Tumor Biology and Translational Medicine. 2013. Curr Protoc Pharmacol.

Lena Biosciences' PerfusionPal System



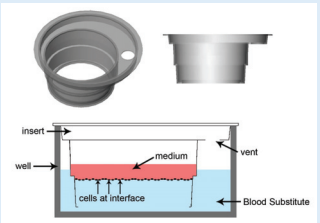
Cells seeded in medium or in 3D hydrogels supported by a rigid scaffold are maintained in statistically independent wells in their own medium which floats atop a high-density blood substitute in which medium and reagents are immiscible. The blood substitute readily dissolves atmospheric gases at levels which are orders of magnitude higher than for aqueous medium. Infusion and withdrawal of this blood substitute acts as a "liquid piston" and introduces perfusion into the system without the need for separate pumps and tubing for each culture condition.

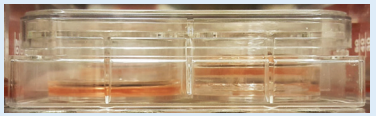
Culturing in PerfusionPal potentiates hepatic CYP activity for drug toxicity testing



Culturing primary human hepatocytes in the PerfusionPal system consistently enhances baseline and induced CYP450 activity. Primary human hepatocytes (BioIVT Cryoplateable 10-donor Liverpool™) were maintained in culture over a 7-day period (n=3 for all conditions) in either a multi-well plate ("2D Control") or in Lena Biosciences' PerfusionPal system ("PerfusionPal") with a perfusion rate corresponding to 16 culture medium volume exchanges per 24-hour period. Baseline CYP activity (left) was significantly increased for all enzymes tested. Induced CYP activity (right) was similarly enhanced by the perfusion system. The uninduced CYP activities of a number of CYP enzymes in PerfusionPal were higher than the induced activities in 2D. Note the log scale for the left graph. All data are normalized to their respective uninduced 2D control. *p<0.05, **p<0.01, ***p<0.001

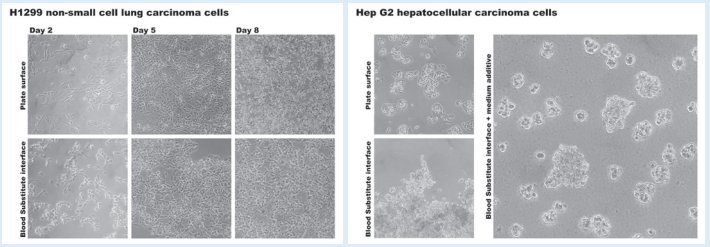
Lena Biosciences' liquid-liquid interface method for scaffold-free 3D cell culture





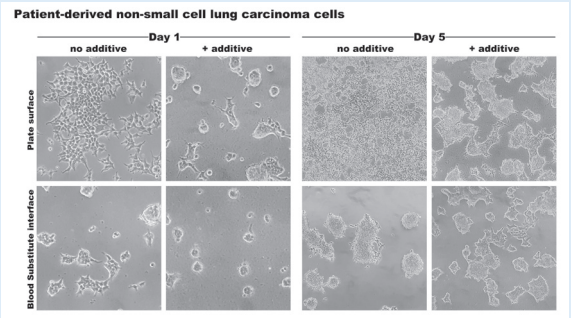
Insert system provides a simple method for culturing cells at a liquid-liquid interface between medium and Blood Substitute. PerfusionPal multi-well inserts (12-well or 48-well) or single 6-well inserts (pictured) can be used with standard multi-well plates with Blood Substitute in the wells. Cells are seeded in medium or medium modified with an additive to promote cell attachment at the Blood Substitute surface.

Cell response to the liquid-liquid interface is cell-type dependent



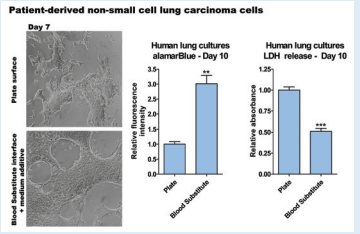
Some immortalized cell lines grown on the surface of the Blood Substitute may require a proprietary additive to facilitate attachment at the interface. H1299 NSCLC cells (left) were grown in a multi-well plate or atop the Blood Substitute in 6-well inserts. Photos were acquired at the time points indicated. On days 2 and 5, cell morphology and distribution were comparable. At day 8, the cells in the multi-well plate had become overgrown and many appeared to be dead. The Blood Substitute may limit this excessive growth through the more physiological delivery of gases such as oxygen. Hep G2 hepatocellular carcinoma cells (right) tended to clump together and die when grown directly on the Blood Substitute. Introduction of an additive to the plating medium facilitated cellular attachment and promoted the formation of spheroids.

Growth of Patient-derived NSCLC cells on Blood Substitute induces spheroid formation



Patient-derived NSCLC cells demonstrated spontaneous spheroid formation on the surface of the Blood Substitute, an effect possibly enhanced by the medium additive. Patient-derived cells were plated in a multi-well plate or atop the Blood Substitute in the presence or absence of the medium additive. On day 1, the effect of the additive was apparent, even in the multi-well plate, as the cells began to form spheroids. Without the additive, the cells grown on the blood substitute also began to form spheroids. On day 5, spheroid growth could be observed in all conditions except the additive-free, multi-well plate condition. The Blood Substitute alone induces spheroid formation, although the presence of the additive seems to result in smaller, more consistent spheroids.

Patient-derived NSCLC cells plated at high density exhibit a tissue-like morphology and increased respiration and viability



High-density plating of patient-derived NSCLC cells on Blood Substitute with medium additive appear to form a more viable, tissue-like 3D construct. An order of magnitude higher density of patient-derived cells were plated in a multi-well plate or atop the Blood Substitute with medium additive (n=6 per condition). On day 7, the cells on the Blood Substitute were noticeably denser, exhibiting a tissue-like appearance. Total cellular respiration as measured by alamarBlue on day 10 showed that the cells on the Blood Substitute were three times more metabolically active than those in the multi-well plate. LDH release from the cells on the Blood Substitute was half that of those in the multi-well plate, indicating higher cell viability. **p<0.01, ***p<0.001

CONCLUSIONS/FUTURE DIRECTIONS

- We have developed a modified application of our PerfusionPal Blood Substitute and multi-well inserts to generate 3D cell cultures without the need for scaffolds.
- The superior gas exchange provided by the Blood Substitute results in a more physiologically relevant environment that facilitates *in vivo-like* spheroid and tissue growth.
- Future studies will incorporate freshly acquired tissue samples to demonstrate maintenance of *in vivo* cell characteristics to validate this system as a model for the study of cancer metastasis and pharmaceutical testing.

This research was supported by an NIH Phase I SBIR award (1R43TR001286-01A1), an NIH Phase II SBIR award (2R44TR001286-02) and an NSF Phase I SBIR award (1549126).