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Epithelial-Mesenchymal Crosstalk Influences Cancer-Related Cell Behavior: A 3D Lung Alveolus-Fibroblast Co-Culture System

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Abstract

Lung cancer is a devastating disease that kills more individuals in the United States than any other cancer. The tumor microenvironment is increasingly recognized as playing a major role in the progression of cancer. Thus, studying the interactions among lung cancer cells, non-malignant cells and the surrounding matrix is critical for understanding and treating lung cancer. Three-dimensional in vitro co-culture systems allow for tissue-relevant platforms that better recapitulate the native cell environment. In this work, we employed a cyst templating technique to culture alveolar epithelial cells on photodegradable microspheres and subsequently encapsulated the cell-covered spheres within poly(ethylene glycol) (PEG) hydrogels laden with pulmonary fibroblasts. A healthy fibroblast cell line was co-cultured with either normal mouse alveolar epithelial primary cells or a cancerous alveolar epithelial cell line to probe the influence of tumor-stromal co-culture on cell behavior. We found that cancerous epithelial cells and normal fibroblasts have significantly higher proliferation rates in co-culture, normal fibroblasts migrate significantly faster when co-cultured with cancerous A549 cells, and there is a synergistic increase in matrix metalloproteinase (MMP) activity in this cancer co-culture. None of these changes in cell behavior were observed in a parallel healthy epithelial-cyst/fibroblast co-culture. Further, when MMP activity was reduced via a chemical inhibitor and when cells were cultured in gels with a non-degradable crosslinker, the increase in cancer cell proliferation in co-culture was abrogated and fibroblast migration was dramatically suppressed. The results presented here suggest a nuanced chemical signaling exchange between lung cancer epithelial cells and pulmonary fibroblasts within our 3D hydrogel system and points to potential signaling routes that merit further investigation.
1. Introduction

Lung cancer kills more individuals in the United States than any other type of cancer, and the survival rate for metastatic lung cancer is less than 4% (US Cancer Stats). Lung cancer is generally classified as either small cell or non-small cell lung cancer (NSCLC). NSCLC accounts for nearly 85% of lung cancers and is generally diagnosed at late, metastatic stages of disease, limiting treatment options and efficacy (Rosell 2013). Although some of these tumors are initially resectable or responsive to conventional therapy, drug resistance and disease recurrence rates remain problematic (Kelsey 2009; Yang 2015).

Researchers are constantly exploring new techniques for treating NSCLC. As disease mechanisms and screening for potential treatments are often studied in vitro, it is essential that the results of preliminary studies are as physiologically relevant as possible. The current standard of studying cells in isolation relies on culturing them on stiff substrates (i.e., tissue culture plasticware) that are immensely different from the native lung environment and is often not representative of their behavior in vivo (Puliafito 2012; Rejniak 2010). In the native lung environment, epithelial cells form polarized, hollow spherical structures with air-sacs on one side and a complex structure of matrix proteins, biochemical signaling molecules, and a variety of other cell types on the other. The complex interactions between epithelial cells and their environment are vital components of both healthy lung physiology and disease progression, and thus should be considered as the field aims to better understand the complex behavior of diseased cells, as well as the interactions between diseased and disease-associated cells.
In this thesis, we aimed to utilize our previously developed photodegradable microgel cyst-templating technique to guide the formation of hollow, multicellular lung epithelial spheres, which mimic the geometry of alveoli in the native lung environment. We encapsulated cell-coated microspheres within a secondary hydrogel laden with pulmonary fibroblasts to study the interactions between the lung epithelium and the surrounding stroma as related to cancer progression in a physiologically relevant geometry. We probed changes in cell proliferation, enzyme activity and gene expression of healthy epithelial cysts, cancer epithelial cysts, and healthy lung fibroblasts, both alone and in co-culture, within this 3D in-vitro microenvironment.

2. Background

2.1 The Alveolar Microenvironment and Cancer

The tumor microenvironment plays an active role in cancer progression (Balkwill 2012). Extracellular matrix (ECM) proteins and non-malignant cells, such as fibroblasts and immune cells within this space, can function to promote tumor invasion and metastasis via biochemical signaling and matrix remodeling (Balkwill 2012). The ECM is a multifaceted complex of proteins that provides structural support for tissues and also serves as a reservoir that sequesters various cytokines and chemokines. It is composed of fibrous proteins, glycoproteins, and proteoglycans that provide both a structural matrix for cells, as well as spatial and mechanical cues that elicit specific cellular responses and phenotypes (White 2015).

In the lungs, a polarized layer of epithelial cells attached to a thin basement membrane form hollow, cyst-like structures called alveoli (Herzog 2008). Interstitial
stromal cells, such as alveolar fibroblasts, surround these structures, and gaps in the basement membrane allow for communication between the epithelium and the interstitial fibroblasts (Herzog 2008, Hogan 2015; Sirianni 2003). These fibroblasts modulate the composition and elasticity of the ECM via protein synthesis and degradation in the healthy lung and during wound repair (White 2015).

In response to lung injury, resident fibroblasts activate and become proliferative, migratory and contractile as they remodel the ECM to allow for wound closure. These myofibroblasts secrete ECM proteins that signal proliferation and differentiation of epithelial cells and provide a structure for cell migration (Selman 2004). While this process is essential for normal tissue repair, disruption of this signaling has been implicated in the development and progression of lung diseases including cancer (Thiery 2009). After a wound is resolved in a normal lung, the myofibroblast population reverts to a quiescent phenotype and the homeostasis of ECM synthesis and degradation is re-established (Marsh 2005). However, during tumorigenesis, tumor secreted factors such as TGFβ, PDGF, and IL-1 may cause the myofibroblast population to persist and transform into cancer-associated fibroblasts (CAFs) that promote tumor growth and disease progression (Marsh 2005, Agusten, 2014).

2.2 Extracellular Matrix Remodeling

Activated fibroblasts secrete matrix-remodeling enzymes that include matrix metalloproteinases (MMPs) and their inhibitors, tissue inhibitors of matrix metalloproteinases (TIMPs). Maintaining a balance between MMPs and TIMPs is essential for normal tissue elasticity and function, and disregulation of this balance may facilitate cancer cell migration and invasive cancer growth (DeWever 2008). It is possible that
paracrine signaling between lung cancer epithelial cells and interstitial fibroblasts leads to an imbalance of matrix turnover and degradation, which favors matrix degradation. This imbalance could allow for increased local cancer cell infiltration, decreased contact inhibition, and eventual metastasis.

2.3 Three-Dimensional Recapitulation of the Alveolar Microenvironment to Study Epithelial-Fibroblast Interactions

A large body of research has been focused on studying the crosstalk between tumor and stromal cells; however, most of these studies have been carried out in conventional 2D monolayers that significantly alter cell morphology and density from their native environments (Alonso-Nocelo 2016; Fromigue 2003). The cellular microenvironment including matrix stiffness, topography, and chemical cues are known to have a substantial impact on cellular behavior, gene expression, and phenotype (White 2015).

Due to the significant contributions of ECM dynamics and epithelial-mesenchymal exchanges in lung disease progression, it is important to study these interactions in a more physiologically relevant *in vitro* culture system (Prasad 2014; Horie 2012; Kim 2015). The elastic modulus of healthy lung tissue has been reported to be within the range of 5-30 kPa, which is far softer (nearly 6 orders of magnitude) than traditional tissue culture substrates such as glass coverslips or tissue culture plastic (Cavalcante 2005). More physiologically relevant systems utilize ECM mimics that allow for culture of multicellular tissue structures where cells can interact with their environment as they might in native tissue. Three dimensional *in vitro* culture systems allow researchers to readily manipulate the environment of the cell type that they are interested in studying. For example, experimenters can precisely control the biochemical signals a cell is exposed to by
entrapping or tethering proteins and small molecules within a hydrogel matrix (Grim 2015). The elastic modulus can also be readily manipulated to better recapitulate in vivo healthy and diseased tissue (Mabry 2016; Lewis 2013). Further, other cell types can be introduced to the system to allow researchers to probe interactions between healthy and diseased cell types, while controlling their density and spatial proximity, as in the work presented in this thesis.

Although techniques to form in vitro 3D tumor spheroids currently exist, most do not adequately recapitulate the hollow alveolus structures that exist within the lung (Friedrich 2007; Seidl 2002; Weiswald 2015). These models, such as the hanging drop method or other methods that produce cancer cell aggregates, generally employ a dense tumor spheroid to study cancer biology. However, these models do not achieve a cyst-like alveolus structure that would be characteristic of lung cells’ in vivo geometry. Further, these methods often do not allow for a relevant comparison to a healthy counterpart, where primary alveolar epithelial cells are used to represent a healthy phenotype.

Our lab has recently demonstrated a technique for producing hollow, multicellular lung epithelial spheres of discrete size ranges within a peptide functionalized poly(ethylene glycol) (PEG) hydrogel using a photodegradable PEG hydrogel template to guide cyst formation (Figure 1, Lewis 2015). This culture system mimics essential features of the native alveolar microenvironment, including appropriate tissue elasticity, protease-degradable hydrogel cross-linkers that enable local remodeling, and integrin binding sites for cellular attachment. This system allows for co-culture of both healthy and diseased epithelial cysts with interstitial cells within a dynamic matrix.
Using this technique, our lab has shown that both non-small cell lung cancer cell line cells (A549) and primary murine alveolar type II cells will attach to the microspheres and proliferate to form hollow, model alveoli (Figure 2A,B). Further, primary cell cysts form normal cell adherens and tight junctions, as evidenced by β-catenin and ZO-1, respectively, and differentiate into a similar distribution of alveolar type I (ATI) and alveolar type II (ATII), as demonstrated by SPC and T1-α staining, as is seen in vivo (Lewis 2015) (Figure 2C).

In this work, primary mouse epithelial cells and an adenocarcinoma cell line (A549) were used to form epithelial cysts, and then co-cultured with a healthy pulmonary fibroblast cell line (CCL-210) to study changes in behavior in response to a healthy and diseased alveolar model.
3. Specific Aims

This thesis research aims to study how extracellular cues influence alveolar epithelial cells and pulmonary fibroblasts in the context of a biologically relevant cellular arrangement. We postulate that the 3D culture system used in this research is robust and versatile for manipulating the multi-cellular architecture of lung cells, and enables us to study epithelial-matrix and epithelial-mesenchymal interactions in a model alveolus microenvironment \textit{in vitro}. To test this hypothesis, the specific aims of this thesis are to:
1. Study changes in the proliferation of healthy lung epithelial cells, lung carcinoma cells and pulmonary fibroblasts in our 3D epithelial-fibroblast co-culture system vs. monoculture.

2. Probe changes in migration of healthy lung fibroblasts in response to 3D co-culture with healthy or carcinoma-based lung epithelial cysts.

3. Determine the effects of co-culture conditions on total metabolic and MMP activity in healthy or diseased lung models.

4. Materials and Methods

4.1 Microsphere synthesis

Photodegradable microspheres were created using an inverse suspension polymerization technique. Particles were polymerized via base-catalyzed Michael addition of a photodegradable diacrylate (PEGdiPDA; Mn~4070 Da) with a poly(ethylene glycol) tetrathiol (PEG4SH; Mn~5000 Da). PEG4SH was purchased from JenKem Technology and PEGdiPDA was synthesized as previously described [30]. An aqueous phase consisting of 6.9 wt% PEGdiPDA, 4.2 wt% PEG4SH, CRGDS peptide (1.5 mM final concentration), 300mM triethanolamine (Sigma-Aldrich) in pH 8.0 phosphate buffered saline (PBS, Sigma-Aldrich) was added and mixed twice via pipetting into an organic phase containing 3:1 sorbitan monooleate (Span 80, Sigma-Aldrich) and PEG-sorbitanmonooleate (Tween80, Sigma-Aldrich) dissolved at 30 mg surfactant per mL in hexanes (EMD Millipore). The droplet suspension was immediately stirred at ~200 rpm in a 20 mL glass scintillation vial with a 1cm magnetic stir bar overnight, protected from light. Microspheres were collected via
centrifugation and washed consecutively with hexanes, isopropanol, and sterile phosphate-buffered saline (PBS).

Fluorescently labeled, non-degradable microspheres were formed using a non-degradable poly(ethylene glycol) diacrylate (PEGDA; Mn~4000) that had been pre-reacted In the dark with alexaFluor-488 C5 maleimide (Life Technologies; <1 mM) for 30 minutes. Microspheres were synthesized as described above, with an aqueous phase comprised of 6.9 wt% PEGDA, 4.2 wt% AlexaFluor-488-labeled PEG4SH, 300 mM triethanolamine in pH 8.0 PBS.

**Figure 3: Microsphere gel formulation and erosion.** (A) The microspheres were composed of 4-arm PEG tetrathiol, a photodegradable 2 arm PEG di-photodegradable acrylate (PEGdiDA) crosslinker and adhesive peptide CRGDS. These monomers were polymerized using a base catalized Michael addition and formed via inverse suspension polymerization. (B) Histogram shows distribution of fluorescently labeled microsphere diameters, and the black line indicates the cumulative percentage of the population. The microspheres have an average diameter of 120 ± 70 µm with a polydispersity of 1.4, and 90% of the particles are <200 µm in diameter. Inset shows representative fluorescent image of microspheres used for diameter measurement. n = 3087 (C) Cleavage of the nitrobenzyl ether moiety with light is shown in the chemical structure, and the absorbance spectrum is for the PEGdiPDA crosslinker (0.8 mM) in phosphate-buffered saline, where the blue line indicates 365 nm. (Adapted from Lewis, et al., Biomater Sci (2015) 3: 821-832)
4.2 Cell culture

Normal human lung fibroblasts (CCL-210, ATCC) were cultured in low glucose Dulbecco’s modified Eagle’s medium (DMEM, Life Technologies) and A549 human adenocarcinoma cells (CCL-185, ATCC) were cultured in high glucose DMEM. Growth medium was supplemented with 10% fetal bovine serum (FBS, Life Technologies), 1% penicillin/streptomycin (Life Technologies), and 0.2% fungizone (Life Technologies) and cells were cultured at 37 °C with 5% CO₂.

4.3 Primary ATII cell isolation

All animal procedures and protocols were reviewed and approved by the Animal Care and Use Committee at the University of Colorado, Boulder. Primary mouse alveolar epithelial type II (ATII) cells were isolated as previously described (Kloxin & Lewis 2012). Male FVB/NJ mice (6 weeks old) were acquired from The Jackson Laboratories and immediately euthanized by CO₂ asphyxiation. The animal’s chest cavity was exposed to visualize the heart-lung block, heparin was injected into the left ventricle prior to cutting the inferior vena cava. The pulmonary vasculature was flushed with 1% heparin in sterile PBS. The heart-lung block was removed, and lung tissue was separated carefully from the heart, trachea, and connective tissue. The remaining lung tissue was thoroughly minced and subsequently digested in 0.1% Type-1 collagenase (Worthington Biochemical) and 1 mg/mL DNase (Sigma) for 20 minutes at 37°C. Trypsin (Fisher, 0.01% final concentration) was added and the solution was further incubated for 20 minutes at 37°C prior to final addition of a 1mg/mL trypsin inhibitor (Life Technologies) and 1 mg/mL DNase solution. This mixture was first filtered through 100 μm cell strainers and then through 10 μm pore Nitex filter.
The resultant solution was centrifuged for 5 minutes at 2000 rpm, and the pellet was resuspended in DMEM/F-12 medium (Sigma). This cell suspension was incubated for one hour on tissue culture plates coated with IgG (11 mg/cm2) to remove contaminating immune cells. Non-adherent cells were recovered, centrifuged for 5 minutes at 2000 rpm and resuspended in 1:1 Dulbecco’s Modified Eagle Medium:Nutrient Mixture F-12 (DEMEM/F12, Corning) supplemented with 10% FBS and 1% antibiotic/antimitotic (Life Technologies) for counting. Cells were finally centrifuged at 2000 rpm for 5 minutes and resuspended at 500,000 cells/mL in DMEM/F-12 medium supplemented with 10% FBS, 1% antibiotic/antimitotic, hepatocyte growth factor (HGF; R&D Systems; 50 ng/mL), and Fibroblast Growth Factor 7 (FGF-7; Sigma; 10 ng/mL) and immediately used in cyst experiments.

4.4 Microsphere seeding

Primary ATII cells at 500,000 cells/mL or A549 cells at 150,000 cells/mL were mixed with 40 μL of photodegradable microspheres in an ultra-low adhesion 24-well plate (Corning). This suspension was incubated at 37°C with 5% CO2 on an orbital shaker at 45 rpm. A549-microparticle suspensions were incubated for 18-24 hours and primary-microparticle suspensions were incubated for 3 days to allow for optimal template coverage and cell attachment (Lewis 2015). Pre-cysts were harvested after the appropriate incubation period and allowed to settle by gravity in a conical tube. The supernatant was removed by careful pipetting and pre-cysts were resuspended in PBS for use in experiments.
4.5 Cell labeling

For proliferation assays and co-culture migration experiments, fibroblasts were labeled with Cell Tracker Green CMFDA (Life Technologies) per manufacturer’s instructions. Fibroblasts were incubated in appropriate serum-free growth medium containing Cell Tracker Green (10 μM final concentration) at 37°C for 30 minutes. Cells were subsequently centrifuged and resuspended in PBS at 5 million cells/mL for encapsulation in hydrogels.

For fibroblast-only migration experiments, fibroblasts were stained with Cell Tracker Red CMPTX (Life Technologies, 20 μM final concentration) as above. For co-culture migration experiments, epithelial pre-cysts were labeled with Cell Tracker Red CMPTX (Life Technologies). Appropriate serum-free growth medium containing Cell Tracker Red (20 μM final concentration) was added to pre-cysts and incubated for 30 minutes at 37°C. Cell Tracker Red medium was removed carefully using a pipette, and cysts were resuspended in PBS prior to encapsulation in hydrogels.

Hydrogel Materials

8-arm poly(ethylene glycol) norbornene (PEG-Nb Mw ~40,000) and photoinitiator lithium phenyl-2,4,6-trimethylbenzoylphosphinate (LAP) were synthesized as previously described (Gould 2009; Fairbanks 2009). An integrin-binding peptide (CRGDS) and an enzymatically-cleavable di-cysteine peptide (KCGPQG↓IWGQCK) were purchased from American Peptide Company, Inc. A non-degradable di-cysteine peptide (KCGPQG↓IWGQCK) containing the D isoform of isoleucine was synthesized using solid phase peptide synthesis on a Tribute Protein Synthesizer (Protein Technologies) with a Rink Amide MBHA resin (Novabiochem), as previously reported (Yang 2012). A quenched MMP-cleavable
fluorogenic peptide substrate (Dabcyl-GGPQGIWGQK-Fluorescein-AEEAcC) was synthesized using solid phase peptide synthesis as previously published (Leight 2013).

Figure 4: Encapsulating Hydrogel Synthesis. The encapsulating gel was composed of 8-arm PEGnb (Mn ~ 40 kDa) crosslinked by an enzymatically-cleavable di-cysteine peptide (KCGPQGIWGQCK) with the adhesive peptide CRGDS included at 1 mM. The arrow indicates the enzymatic cleavage site. Thiol groups (red) react with the -ene functionalities on the 8-arm PEG through a radical-initiated thiol–ene polymerization. (Adapted from Lewis, et al., Biomater Sci (2015) 3: 821-832)

4.6 Proliferation Hydrogel Formation

For Click-iT Plus EdU assays, 8-arm 40 kDa PEG-Nb (5 wt%) was combined with di-cysteine peptide crosslinker (degradable or non-degradable) (0.85:1 thiol:ene), CRGDS (1mM), and LAP (0.05 wt%) in sterile PBS and mixed by vortex to form a gel precursor solution. Sterile 0.1 M sodium hydroxide was used to adjust the pH between 6.8-7.2. Epithelial cysts and/or a fibroblast cell suspension (final concentration 1.6 million cells/mL) were added to the gel precursor solution and gently mixed with a pipette. 30 μL drops of this precursor solution were placed on Sigmacote-treated glass slides and exposed to 365 nm light at ~2 mW/cm² for 3 minutes to initiate the radical-mediated thiol-ene
polymerization reaction. Polymerized, cell-laden hydrogels were transferred to an untreated 24-well plate (Corning) containing 1 mL of DMEM/F-12 growth medium supplemented with 10% FBS and 1% antibiotic/antimitotic and incubated at 37 °C with 5% CO₂. For MMP inhibition studies, 10 μM GM6001 in DMSO (Santa Cruz Biotechnology) or 0.05% DMSO was added to the media.

4.7 Migration Gels

8-arm 40kDa PEG-Nb (3 wt%), di-cysteine MMP-degradable crosslinker (0.75:1), CRGDS (1 mM), and LAP (0.05 wt%) were combined in sterile PBS and the pH was adjusted using sterile sodium hydroxide (0.1 M) to 6.8-7.2. A fibroblast cell suspension (final concentration 1 million cells/mL) and either epithelial cysts or fluorescently-labeled non-degradable microspheres were added to the gel precursor solution and gently pipetted to mix.

A 24-well glass-bottomed plate (Greiner Bio-One) was surface functionalized with thiol groups to allow for covalent hydrogel attachment. Plates were washed in 95% ethanol before functionalization in 0.5% (v/v) (3-mercaptopropyl)trimethoxysilane in 95% ethanol (pH ~5.5) for 5 minutes. Each well was washed again with 95% ethanol and allowed to air dry. A biopsy punch was used to form 6 mm diameter circles was cut within square, 1 mm tall rubber gaskets. The gaskets were sealed to the bottom of the wells in the thiolated 24-well glass-bottom plate and 30 μL drops of the gel precursor solution were added to the gaskets. The plate was exposed to 365 nm light at ~2 mW/cm² for 4 minutes to initiate radical mediated polymerization. Rubber gaskets were carefully removed from polymerized hydrogels and 1mL of DMEM/F-12 growth medium supplemented with 10% FBS and 1% antibiotic/antimitotic was added. Gels were incubated at 37 °C with 5% CO₂.
For MMP inhibition studies, 10 μM GM6001 (Santa Cruz Biotechnology) was added to the media.

4.8 MMP Sensor Peptide Gels

A quenched, MMP-cleavable fluorogenic peptide substrate with the same sequence used to crosslink the hydrogels (Dabcyl-GGPQG↓IWGQK-Fluorescein-AEEAcC) was incorporated into the hydrogel network as described previously to evaluate MMP activity (Leight 2013; Leight 2015). To form MMP sensor gels, 8-arm 40k PEG-Nb (5 wt%), was combined with di-cysteine MMP-degradable peptide crosslinker (0.85:1 thiol:ene), fluorogenic MMP sensor peptide (0.25 mM), CRGDS (1 mM), and LAP photoinitiator (0.05 wt%) in sterile PBS. The pH was adjusted to 6.8-7.2 with sterile, 0.1 M sodium hydroxide. Cells and/or cysts were added to the gel precursor as previously described.

To allow for a covalent linkage between the hydrogel and coverslip, hydrogels were polymerized on top of thiol-functionalized glass coverslips (12 mm, Fisher Scientific). Glass coverslips were flame cleaned and thiol-functionalized via liquid deposition in silane solution (0.5% (3-mercaptopropyl) trimethoxysilane (Sigma Aldrich)) in 95% ethanol/water, pH~5.5). Coverslips were rinsed in 95% ethanol in water, and dried. 50 μL of precursor solution was pipetted into a rubber gasket with a 6 mm inner diameter centered on a functionalized coverslip and polymerized under 365 nm light at an intensity of ~2 mW/cm² for 3 minutes. Rubber gaskets were removed and gels covalently attached to coverslips were transferred to a 24-well plate containing 1 mL DMEM/F-12 growth medium. Culture media was changed one hour after hydrogel polymerization to remove any excess fluorogenic peptide. Gels were incubated for 22 to 46 hours after encapsulation.
For MMP inhibition studies, 10 or 100 μM GM6001 (Santa Cruz Biotechnology) or 0.5% DMSO was added to the media.

4.9 Microparticle template erosion

Gels containing fibroblasts and/or cysts were cultured for one day to allow cell attachment to the encapsulating hydrogel. Following this 24-hour incubation period, all samples were exposed to 365 nm light at ~10 mW/cm² for 15 minutes to cleave the photolabile moiety in the microspheres and fully erode the cyst templates. Gels were then incubated for ~1 hour at 37°C with 5% CO₂ before changing growth medium. Growth medium was then exchanged daily until completion of each experiment (between 1 and 5 days).

4.10 Click-iT EdU assay and quantifying proliferation

Cell proliferation in three biological replicates of each cell combination was detected using a Click-iT Plus EdU AlexaFluor-594 imaging kit (Life Technologies). A copper-catalyzed covalent “click reaction” between a picolyl azide on an AlexaFluor dye and an alkyne on the thymidine nucleoside analog, 5-ethynyl-2’-deoxyuridine (EdU), allowed for fluorescent staining of proliferating nuclei. EdU was added to growth media at a final concentration of 10 μM on day 1 or day 4 after cell/cyst encapsulation. Samples were then incubated for 17 hours at 37°C with 5% CO₂ to allow for EdU incorporation into DNA during active DNA synthesis, per manufacturer instructions. Samples were then fixed with 4% paraformaldehyde in PBS for 15 minutes at room temperature and washed in 3% bovine serum albumin (BSA) in PBS. Cells were permeabilized in 1% TritonX-100 for one hour and washed with 3% BSA in PBS. Click-it Plus reaction cocktail containing the AlexaFluor 594 dye was prepared as per manufacturer instructions, added to each sample,
and incubated for 3 hours at room temperature, rocking. Samples were then washed with 3% BSA in PBS. Finally, nuclei were stained with DAPI (1:2000, Life Technologies) for one hour and washed in PBS. All samples were imaged on a confocal microscope (Zeiss LSM 710) with a 20× water-dipping objective (Plan Appochromat; NA = 1.0). Image stacks (10 per condition) were taken from the top to bottom of individual cysts, or in the center of the gel for fibroblast-only conditions (z step = 10 μm, average stack size ~500 μm).

For quantification, four categories of cell nuclei were counted using MATLAB: fibroblast nuclei, epithelial nuclei, proliferating fibroblast nuclei, and proliferating epithelial nuclei. TIFF images of each z-slice in each of three channels: DAPI, AF-594, and Cell Tracker Green, were created in Image J and processed in MATLAB. Separate masks were made of objects (nuclei) in the DAPI channel and AlexaFluor 594 channels, and objects were dilated by two pixels with a Disk Structuring Element. The mean intensity within each dilated object in the Cell Tracker Green channel was evaluated. Objects with a mean intensity above a given threshold were counted as fibroblasts; if the mean intensity fell below this threshold, the object was counted as an epithelial cell. Cell nuclei centroids were tracked and checked for their appearance in successive slices to account for double counted nuclei. Double counts were subtracted to give final cell counts and the percent of proliferating cells. Object counts were pooled from all ten image stacks, and the mean percent positive for EdU was calculated from three biological replicates. Statistical analysis was performed using two-way ANOVA followed by Bonferroni posttests. All error bars represent standard error of the mean (SEM).
4.11 Tracking and quantification of cell migration

The migration of fibroblasts in co-culture hydrogels was tracked with an Operetta High Content Imaging System (Perkin Elmer) with Harmony High Content Imaging for automated image analysis in real time. A live cell chamber was used to maintain the temperature at 37 °C and 5% CO₂ for the duration of a 24 hour experiment. The 10x long WD objective was used with the optical model set to confocal. AlexaFluor 594 and AlexaFluor 488 channels were selected and exposure times were optimized for each experiment. Nine fields of view per well were imaged at 30-minute intervals within a 3x3 square in the center of the well. 400 μm z-stacks beginning at a height of 25 μm from the glass surface were collected for each field of view.

Fibroblast migration was analyzed using Volocity 3D Image Analysis Software (Perkin Elmer). The protocol tracked fibroblasts (AlexaFluor 488 for cyst co-cultures, AlexaFluor 594 for fibroblast-only gels) within a spherical region of interest (ROI) around a cyst or fluorescent microsphere. Either an epithelial cyst (AlexaFluor 594 channel) or a fluorescent microsphere (AlexaFluor 488 channel) was tracked as a single object to be used as a reference point. Only fibroblasts within a 125 μm radius outside of the reference object were analyzed in order to limit ROI overlap between neighboring cysts/microspheres, which were spaced 250 μm apart on average.

The centroid data of the tracked reference objects and individual fibroblasts was exported and used to determine cell speeds, displacements, directionality, and the percent of migrating cells via MATLAB analysis. To compensate for drift, the fibroblast centroid data was normalized to the reference object centroid. Fibroblast speeds were calculated as the average of the individual speeds between subsequent time points on the cell path.
Displacement was calculated as the distance between start and end points, and was used to indicate if a fibroblast was migrating towards or away from a cyst. A positive displacement indicated movement towards a cyst and negative displacement indicated movement away from a cyst. The directionality, as defined as displacement over total distance traveled, measured the straightness of a cell path analogous to persistence time, with values close to 1 indicating a perfectly linear path (Schwartz 2013). The fraction of migrating fibroblasts was defined as the number of migrating fibroblasts divided by the total number of fibroblasts tracked. A cell was deemed migrating if the maximum distance away from the starting position was greater than one cell body length (15 μm) at any point during the 24 hour period. Statistical analysis was performed using the Kruskal-Wallis test followed by Dunn's Multiple Comparison test. All error bars represent standard error of the mean (SEM).

4.12 Quantification of MMP activity and metabolic activity

After 22 or 46 hours post-encapsulation, 10% PrestoBlue (Invitrogen) was added to each well to detect metabolic activity. The reduction of resazurin (blue and non-fluorescent) to resorufin (red and fluorescent) in the cell cytoplasm is proportional to the number of metabolically active cells and can be measured quantitatively by a change in fluorescence. Two hours after addition of PrestoBlue, fluorescence intensity readings were conducted using a Synergy H1 microplate reader (BioTek) at 560 nm excitation/590 nm emission for PrestoBlue, and 494 nm excitation/521 nm emission for the fluorogenic peptide. After fluorescence readings, cells were immediately fixed with 4% PFA for 15 minutes and washed thrice with PBS. Cells were then stained with Hoechst (1:1000, ThermoScientific) for 1 hour and subsequently washed with PBS.
Gels were imaged using an Operetta High Content Imaging System (Perkin Elmer) using Harmony High Content Imaging software, with the 10X long WD objective in confocal mode. 300 μm z-stack images were taken (31 images 20μm apart) in 9 fields of view for each gel. Harmony Analysis software was then used to count cell nuclei. Fluorogenic peptide signal (MMP activity) and PrestoBlue signal (metabolic activity) were each normalized to cell number. Statistical analysis was preformed using two-way ANOVA with Bonferroni posttests. Error bars represent standard error of the mean (SEM).

**Figure 5:** (A). Sequence of MMP degradable peptide (↓ indicates cleavage site). Cleavage of the MMP linker separates the quencher (red circle), dabcyli (Dab), from the fluorophore(green circle), fluorescein (Fl), allowing excitation of the fluorophore. (B) Schematic of flourogenic peptide measurements. Prior to cleavage, the fluorogenic substrate is tethered within the hydrogel matrix, as reflected by a microplate reader area scan of a PEG-NB hydrogel. After degradation, the MMP fluorogenic peptide and the hydrogel structure are degraded leading to quencher separation and diffuse fluorescence.

*Note: The schematic displays a 4 arm PEG system, but the hydrogels used in the work presented here were composed of 8-arm PEG units. (Adapted from Leight, J et al. *Biomaterials* 34, 7344–7352 (2013)).*

5. Results:

Using our cyst-forming technique described in Section 4.4/4.5, we created a 3D *in-vitro* co-culture system to probe the interactions between healthy pulmonary fibroblasts and lung epithelial cysts in comparative recapitulations of the healthy and diseased alveolar microenvironment. Using this system, we studied changes in cellular behavior in
response to co-culture within an appropriate spatial context. Changes in cell proliferation, motility, and MMP and metabolic activity were studied. To mimic both a healthy and diseased environment, three cell types were used: healthy pulmonary fibroblasts (CCL-210 cell line) healthy alveolar epithelial cells (primary mouse cells), and lung tumor epithelial cells (A549 cell line)

5.1 Proliferation

To study cell proliferation, we used a commercially available EdU assay to fluorescently label proliferating cells over a 17 hour period. Proliferation was quantified via automated MATLAB image analysis after 1 and 4 days in 3D culture (Fig. 6A).

Regardless of the condition, all epithelial cells demonstrated a significant decrease in proliferation between day 1 and day 4, (Fig. 6B). Primary epithelial cell proliferation did not change between co-culture and monoculture on either day 1 (~28% positive) or day 4 (~15% positive). On day 1, the A549 cancer cell proliferation also did not significantly change between monoculture (~61% positive) and co-culture (~55% positive). However, at day 4 the A549 proliferation increased when co-cultured with fibroblasts (~45% positive) compared with monoculture (~19% positive). Further, A549 cells demonstrated significantly higher proliferation than primary epithelial cells on day 1 of monoculture and co-culture. On day 4, A549 proliferation was only significantly higher than primary cell proliferation in co-culture conditions.

The fibroblasts were much less proliferative than either epithelial cell type, and their monoculture proliferation rate was relatively consistent over time (Fig. 6B). However, when co-cultured with A549 cells, the fibroblast proliferation was significantly increased on both days (~12% positive) compared to monoculture (~3% positive). A significant
increase in proliferation in the cancer co-culture was also seen when compared to fibroblasts co-cultured with primary cysts on day 4 (~7% positive). Co-culture with primary cysts did not significantly change fibroblast proliferation compared to monoculture.
Figure 6: Click-iT Plus EdU proliferation assay. (A) Example confocal image slices used for MATLAB cell counts. Each object recognized in both blue (DAPI) and red (Edu) channels was measured for mean intensity in the green channel (Cell Tracker), and objects above a certain threshold were counted as fibroblasts. (i) all nuclei, (ii) proliferating nuclei (iii) fibroblasts (iv) Merged image of all channels. (B) Percent of proliferating cells as a percent of total nuclei positive for EdU at day 1 and day 4 in monoculture and co-cultures. Results are presented as means ± SEM of three experimental replicates of each condition. *p < 0.05, **p < 0.01, ***p < 0.001

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5.2 Fibroblast Migration

We also studied changes in various motility parameters of fibroblasts in monoculture and healthy and diseased co-cultures. Increased cell motility has been used as an indicator of disease progression (Schwartz 2014). Evidence supports that tumor cells undergo an epithelial-to-mesenchymal transition (EMT) during which they migrate through the ECM and eventually infiltrate the blood stream, leading to cancer metastasis. These tumors may also encourage transformation of resident fibroblasts into activated cancer associated fibroblasts (CAFs), persistently activating the wound healing response (Thirey 2009). These activated CAFs may help facilitate cancer cell migration through the matrix (Aguste 2014; Scott 2010). Thus, changes in fibroblast migration measurements may suggest cell activation and potential CAF transformation, and epithelial migration may indicate a facilitation of tumor metastasis.

For these studies, cells were tracked over a 24 hour period using live cell confocal microscopy in 3D, and automated image analysis software and codes were used to track cell centroids with respect to a reference fluorescent microsphere in co-cultures or an epithelial cyst in co-culture.

The percent of migrating cells was calculated by counting the number of cells that moved greater than one cell body length (15um) relative to the total cell population, and this was monitored throughout the duration of the experiment. The percentage of migrating cells was between 40-50% of fibroblasts across all conditions (Fig7, B). No significant movement of the epithelial cells was detected in this matrix system. The remaining parameters were calculated based only on migrating cells and included: migration speed, directionality, and the fraction of cells migrating towards the cysts.
The fraction of cells migrating was relatively constant across all conditions, and the motile population did not seem to move preferentially towards or away from the cysts or microspheres (Fig7, B). Although the total number of cells migrating did not change, fibroblasts migrated significantly faster in co-culture with each epithelial cell type (10 μm/h with primary cysts and 15 μm/h with A549 cells) as compared to monoculture (~6 μm/h). While these speeds were slower than has been reported for human dermal fibroblasts (~40 μm/h), they are similar to speeds reported for human mesenchymal stem cells (~5-20 μm/h) within hydrogels similar to those presented here. We also investigated directionality of the cells movement. Directionality was calculated as the distance from the cell’s origin over the total distance that the cell traveled during a 24 hour period. Using this method, a value of 1 would indicate a perfectly straight path and numbers approaching zero would indicate more random migration. The fibroblasts in all conditions displayed relatively random movement (less than 0.26). However, fibroblasts co-cultured with A549 cells displayed significantly more random, or less directional, motion (0.07) than in co-culture with primary cysts (0.17) or in monoculture (0.26).
Fig. 7 Fibroblast migration analysis. (A) Example images of fibroblasts (green) in co-culture with epithelial cysts (red) used in cell tracking. (i) Max z-projection of 400 μm confocal stack. (ii) 3D rendering of 200 μm confocal stack using Volocity (Perkin Elmer). (B) Plots show fraction migrating, fraction migrating toward cyst, migration speed, and directionality (Distance-To-Origin/Total Distance) of migrating normal fibroblasts (CCL-210) in monoculture and co-culture with both epithelial cell types. Data in fraction migrating plot represent means ± SEM of cells migrating per cyst. Data in the remaining plots represent means ± SEM of all migrating cells. ***p < 0.001
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5.3 MMP and Metabolic Activity

Regulation of ECM turnover is essential for tissue maintenance and wound repair. In cancer, an increased level of secreted matrix metalloproteinases and decreased secretion of their inhibitors (TIMPs) favors matrix degradation and has been implicated in increased tumor cell invasion and angiogenesis. To study changes in MMP activity in our hydrogel system, we used a fluorogenic MMP-cleavable peptide (Dabcyl-GGPQG↓IWGQK-Fluorescein- AEEAcC) covalently linked within the hydrogel network. Upon proteinase cleavage, a quencher is separated from the fluorescent moiety, allowing for in situ measurement of global MMP activity using a plate reader (Leight 2013; Leight 2015). Global metabolic activity was measured concurrently with a resazurin assay. The fluorescence measurements from the MMP sensor peptide and the metabolic activity assay were each normalized to estimated cell count based on an automated count of DAPI labeled nuclei. Co-culture values were compared to a calculated weighted average of the two cell types cultured alone. The weighted average represents the level of metabolism or MMP activity that would be expected in the co-culture if no crosstalk occurred between the cell types (Figure 8 represented by the dotted line).

Global metabolic activity appeared to be substantially higher in both the healthy and diseased co-culture at both time points, as compared to the weighted average calculation (Fig 8 A,B). Although both co-cultures demonstrated increased metabolic activity, only the diseased co-culture displayed increased MMP activity as compared to the weighted average (Fig8 C,D). The healthy cell co-cultures demonstrated an average normalized MMP activity of 0.08 on Day 1 as compared to a weighted average of 0.07, and 0.11 on day 2 as compared to 0.10. However, the cancer cell co-cultures had MMP activities of 0.05 on day 1 as
compared to a weighted average of 0.03 and a 0.07 on day 4 as compared to 0.04. Further, the MMP activity on a per-cell basis was significantly higher in the cancer co-culture than in the A549 cyst monoculture (Fig 8, D). This trend was not observed for the healthy co-culture. In fact, on day 1 the MMP activity on a per-cell basis in primary co-culture was significantly lower than in the primary cyst monoculture (Fig 8, C). It is, however, important to note that the primary epithelial cysts demonstrated higher baseline MMP activity in monoculture than the cancerous A549 cysts in monoculture.
Fig. 8  Metabolic activity was measured on days 1 and 2 after encapsulation via resorufin fluorescence normalized to cell count for primary (A) and cancer (B) co-cultures as well as each cell type alone. MMP activity was measured days 1 and 2 after encapsulation by an MMP-sensitive fluorescent sensor peptide normalized to cell count for primary (C) and cancer (D) co-cultures as well as each cell type alone. Dotted lines represent the weighted average of the two cell types cultured alone, which would be the expected value for the co-culture barring any crosstalk. Data represents means ± SEM for 3 biological replicates of each condition. *p < 0.05, **p < 0.01, ***p < 0.001

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5.4 Matrix Metalloproteinase Inhibition

To probe whether or not the observed changes in cell proliferation or migration speed might be explained by changes in MMP activity and hydrogel degradation, we repeated these experiments using a broad-spectrum MMP inhibitor (GM6001) in MMP-degradable hydrogels. GM6001 binds MMP zinc active sites and inhibits the bioactivity of MMPs 1, 2, 3, 8, and 9. To verify that GM6001 would function to decrease MMP activity in our hydrogel system, we performed the MMP activity assay with 10 μM GM6001 in the media. The normalized MMP activity in the GM6001 condition was half of that measured in the DMSO control (Supplementary Fig. 2).

We quantified the proliferation of cancer cysts in co-culture with fibroblasts in MMP-degradable hydrogels with 10 μM GM6001 added to the media and compared these results to a DMSO control group. We also performed the same proliferation assay encapsulating cells in non-degradable hydrogels, which were cross-linked with a peptide with the same sequence as the degradable hydrogels, but the isoleucine at the cleavage site was replaced with D-isoleucine, which is unrecognizable to MMPs.

The MMP inhibitor significantly attenuated increases in A549 proliferation observed on day 4 during co-culture. The A549 cells co-cultured with the MMP inhibitor (~24% positive) returned to nearly the same level of proliferation as was observed in monoculture (~19% positive) (Fig 9). Similarly, non-degradable gels also attenuated increases in A549 proliferation on day 4 (~29% positive). The DMSO control condition (~43% positive) showed no difference from the initial co-culture (~45% positive). The cancer cyst monocultures demonstrated statistically similar levels of proliferation in all conditions (~22% positive).
The corresponding fibroblast proliferation on day 4 in the MMP-inhibited, non-degradable and DMSO control co-cultures indicated no change from the original degradable co-culture gels (Supplementary Fig. 3). The use of either the non-degradable hydrogel formulation or the MMP inhibitor was sufficient to suppress all cell migration in the matrix, so no data related to speed or directionality of the cells could be measured in these gels.

**Fig. 9** Click-iT Plus EdU proliferation assay. Plot shows percent of proliferating A549 cells as percent of nuclei positive for EdU on day 4 in monoculture and the A549/CCL-210 co-culture, separated by gel type and treatment condition. The nondegradable gels contained a peptide crosslinker insensitive to MMP cleavage. GM6001 was added at 10 μM, and the DMSO control media contained 0.05% DMSO. The degradable bars refer to the original experiment in MMP-degradable gels with regular growth media. Results are presented as means ± SEM of three biological replicates of each condition.

*p < 0.05
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6. Discussion:

Interactions between the alveolar lung epithelium and the surrounding stroma are essential in the regulation of lung homeostasis and disease (Hanahan 2012; Kim 2015, DeWeaver 2008; White 2015). A mounting body of evidence indicates that changes in signaling between the alveolar epithelium and interstitial fibroblasts may lead to the progression of multiple lung pathologies (Horie 2012; Augusten 2014). Chemical cues reciprocally exchanged between various cell types as well as mechanical cues from the surrounding extracellular matrix lead to specific cellular phenotypes in vivo, and thus should be fully considered when probing biology in vitro (Lewis 2013; Seidl 2002; Kloxin 2009; Gould 2012). For example, Mabry et al. demonstrated through microarray data that cells cultured in 2D had distinct gene transcription profiles compared to those in 3D hydrogels, and Seidl et al. demonstrated changes in cancer promoting mRNA expression in a cancer co-culture model. (Mabry 2016; Sidel 2002). Tissue-specific culture platforms are therefore essential in gaining a refined understanding of lung biology that takes into account this interplay. Three-dimensional culture methods more closely resemble the microenvironment of cells in vivo and hold particular relevance when studying contact-inhibited growth and cell migration as in this work. The 3D model of distal lung architecture employed here allows for the co-culture of hollow epithelial spheres with dispersed pulmonary fibroblasts within a cell-tunable matrix.

Increases in both epithelial cell and fibroblast proliferation in the lung are indicators of disease progression (Tiery 2009; Bhowmick 2004). Healthy lung epithelium turnover is slow, and has been approximated to take nearly one month (Fehrenbach 2001). However, during wound healing, lung epithelial cells become proliferative to repopulate the wounded
area (Guillot 2013). Similarly, quiescent fibroblasts generally display very low levels of proliferation, and upon activation become proliferative (Kendall 2014). Tumors have been equated to wounds that never heal (Marsh 2005). During normal wound repair, fibroblasts deactivate and cell cycle arrest ensues. However, tumors seem to constantly stimulate the wound response, causing persistence of the myofibroblast population (Marsh 2005).

In our co-culture system, we observed relatively low levels of proliferation in the healthy monoculture gels (i.e., primary epithelial cells and CCL-210s), which did not change during healthy co-culture conditions. These relatively low levels of proliferation represent what one might expect in a healthy lung. However, in the cancer co-cultures, we observed a reciprocal increase in proliferation between healthy lung fibroblasts and cancer epithelial cells, potentially indicating tumor-promoting crosstalk and activation of the wound healing response. Increased proliferation is one marker used to distinguish CAFs from quiescent, normally functioning fibroblasts. Therefore, it is possible that the cancer cells are secreting pro-fibrotic or pro-inflammatory signals such as TGF-β or IL-1 that encourage activation of the normal fibroblasts. After 4 days in monoculture, A549 cancer cell proliferation was significantly decreased, but in co-culture with fibroblasts, their high levels of proliferation were sustained. A possible explanation for this result is that the increased MMP activity observed in this co-culture condition (i.e., A549 with CCL-210) accelerates matrix degradation and allows more space for the cells to grow outward into the matrix. In monoculture, contact inhibition by the surrounding hydrogel matrix may limit epithelial cell proliferation over time, whereas the increased MMP activity observed in co-culture may remove this barrier to growth. This hypothesis was supported by the results from the MMP inhibition experiments, as well as with those using the non-degradable gel
formulations. In both cases, the inability of the cells to degrade the hydrogel matrix led to decreased proliferation levels, similar to those observed in monoculture and healthy co-culture at day 4.

Cell migration can also be an indicator of disease progression (Bhowmick 2004; Guillot 2013). During cancer, the pro-fibrotic and pro-inflammatory signals secreted by tumor cells are thought to contribute to sustaining an activated fibroblast population that displays increased migration, proliferation, and reciprocal secretion of tumor-promoting signals (Bhowmic 2004; Agusten 2014; Marsh 2005). In our hydrogel matrices, we observed an increase in the migration speed of normal fibroblasts when co-cultured with cancer epithelial cells, especially compared to those in monoculture or when co-cultured with healthy epithelial cells. It is possible that the increase in MMP activity helps facilitate increased migration speeds observed in the cancer co-cultures. The persistent movement of these cells was less directional than in monoculture. Multiple factors, including the topography of the ECM and cell adhesion, can influence directionally persistent migration (Shao 2008). Thus, changes in the structure of the gel matrix due to MMP activity in the co-culture condition may have influenced the directionality of the fibroblast migration. However, since the MMP inhibition and non-degradable gel migration experiments effectively blocked all migration, the specific relationship between MMP activity and migration speed and directionality remains elusive.

The balance between MMPs and their inhibitors is essential in the maintenance of healthy tissue structure and elasticity. Significantly altered expression of MMPs and TIMPs has been observed in tissue from NSCLC patients compared to healthy lung tissue (Safrenk
TIMPs inhibit MMPs in a 1:1 manner that alters the rate of enzymatic matrix cleavage; thus, the balance between MMPs and TIMPs determines the overall breakdown of the extracellular matrix, and mis-regulation of MMP/TIMP activity can result in either fibrosis or excessive matrix degradation (Visse 2003). With our MMP-degradable hydrogel, not only is the degradation of the material altered, but also the physical properties of the matrix (e.g., stiffness). An imbalance favoring matrix degradation has been associated with cancer cell invasiveness, angiogenesis, and eventual metastasis (Visse 2003).

In the studies presented here, an increase in total MMP activity was observed when cancer-derived epithelial cysts were co-cultured with pulmonary fibroblasts when compared to the level observed for each cell type in monoculture. This synergistic increase in MMP activity was not observed in the co-culture of healthy epithelial cysts with the pulmonary fibroblasts. This finding suggests that the cancer cells may be secreting pro-inflammatory signals to trigger this increase in matrix degradation, thus promoting disease progression, potentially through mechanisms that influence cell proliferation and migration.

The observed increase in cell proliferation, as well as increases in global MMP activity, in the cancer co-culture points towards a pro-cancer signaling exchange between initially healthy fibroblasts and cancer epithelial cells that is not observed in the parallel healthy co-culture model. Increased fibroblast proliferation, migration speed, and global MMP expression in the cancer co-culture indicate early signs of transition into a cancer associated fibroblast phenotype. While many of the results presented here are in agreement with other previously published works, this system allows researchers to more
directly discern the interactions among variables rather than studying each data set in separate cellular environments. For example, although changes in MMP expression and up regulation of factors that may increase proliferation have been detected in other co-culture models in 2D using outputs such as PCR or immunohistochemistry, our system allows one to observe the interactions among variables in real time in a way that other culture models do not allow. For example, Fromigué, et al. found an increase in the expression levels of MMPs (2, 9, and 11) from direct, 2D co-cultures of A549 cancer cells and CCL-210 pulmonary fibroblasts, which is in alignment with our observation of increased general MMP activity for co-cultures of the same cell lines (Fromigue 2003). However, such 2D studies are limited in their ability to relate these findings within a physiologically relevant space in the context of contact inhibited proliferation or cell motility through a matrix. Using our biomaterial 3D matrix system, we are able to show the interplay among these variables, such as the association between increases in matrix degradation and increased cell proliferation and fibroblast motility, in a controlled, relevant cellular environment.

Further, this system presents a potential method for drug design and testing within a complex biological system that may better represent cell behavior in vivo. The work presented here points to MMPs as influential players in early pathological signaling between cancer epithelial cells and healthy fibroblasts, and MMP inhibitors are currently being tested as anti-cancer therapeutics. However, cellular behaviors targeted by anti-cancer drugs, such as matrix remodeling, contact dependent proliferation and 3D migration, intrinsically require the use of 3D, compliant environments that allow for co-culture of multiple cell types when testing the value of drugs. This co-culture model complements and expands existing lung culture systems by providing a spatially-organized, tissue-
relevant platform that may prove useful for screening of identified therapeutics before moving on to more expensive and complex animal models.

7. Future Directions and Preliminary Data

The results presented here suggest a nuanced chemical signaling exchange between lung cancer epithelial cells and pulmonary fibroblasts within our 3D hydrogel system. While 3D co-culture models provide many benefits for capturing aspects of the physiological environment over their 2D counter-parts, they also introduce complexity when trying to capture cells, sort them, and analyze contributions from individual cell populations (e.g., trophic factors). In an effort to elucidate the specific changes occurring in gene expression from the lung epithelial cells versus fibroblasts used in our model, we have been working to develop methods to isolate, sort and employ quantitative reverse transcriptase PCR (qRT-PCR) to characterize molecular changes in the two cell types as a result of the co-culture. To achieve this, after 2 days of 3D culture, the encapsulating hydrogel will be fully degraded using a collagenase solution to liberate the cells. Cells will then be collected and stained with a monoclonal TE-7 antibody (Millipore), a marker of fibroblasts, and sorted using a FACS technique and immediately lysed in TriReagent for gene expression analysis. The TE-7 antibody was raised against whole human thymic stoma cells and reacts with fibroblasts in tissue as well as cultured fibroblasts (Hanes 1984). RNA will be extracted via standard phenol-chloroform extraction, and cDNA created using a SuperScript III First-Strand Synthesis kit for RT-PCR (Invitrogen) per manufacturers recommendations with Oligo dT primers. The RTq-PCR sample pates will be prepared with SYBR Green supermix (BioRad), 3.5 ng cDNA, and 500 nM primer mixes.
Values will be normalized to those of TATA box binding protein (TBP) using a ΔΔcq method.

### TABLE 1: RT-qPCR Primer Sequences

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<th>Gene</th>
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Preliminary results using this technique suggest an increasing trend in MMP-2, MMP-11, and TGFβ, expression in A549 in co-culture as opposed to monoculture (Fig 10). Further, MMP2 and TGF-β expression also seem to be increased in the CCL-210 cells in co-culture over monoculture (Fig 10). TGF-β signaling pathways mediate many cancer-related biological processes, including cell proliferation, differentiation, angiogenesis, apoptosis, and extracellular matrix remodeling. Over expression of TGF-β also promotes tumor invasiveness and metastasis and has been implicated in Epithelial to Mesenchymal Transition (EMT) (Xiao 2010). Additionally, TGF-β signaling induces MMP expression, and in turn MMPs produced by either cancer cells or stromal cells activate latent TGF-β in the extracellular matrix, further enabling tumor progression (Krstic 2014). As discussed previously, MMP activity has also been closely related to tumor invasiveness, metastasis and proliferation. This synergistic increase in expression of MMPs and TGF-β could support the conclusion that signaling within this alveolar-fibroblast co-culture promotes expression of genes that contribute to the wound-healing response, promote cell proliferation and the transition of healthy fibroblasts into a CAF phenotype.

Tissue Inhibitors of Metalloproteases (TIMPs) are also important mediators of
extracellular matrix remodeling and have important roles outside of their MMP inhibition activity. TIMP-2 has been shown to stimulate the proliferation of normal fibroblasts via cAMP signaling (Corcoran 1995). Higher levels of TIMP2 have also been detected in CAFs that correlated with higher prevalence of distant metastasis. Contrastingly, TIMP-2 has also been shown to mediate inhibition of tumor growth via modulation of both tumor cells and the tumor microenvironment (Bourboulia 2015). Both overexpression and reduction of TIMP2 have been documented in a large number of cancer studies including breast, lung, gastric, and colorectal cancers (Jackson 2016). A decreasing trend in TIMP-2 expression was observed in the A549 cells in co-culture as compared to monoculture (Fig 10), potentially indicating a decrease in TIMP-2’s anti-proliferative effect leading to increased proliferation in co-culture. Interestingly, an increase in TIMP-2 expression was observed in the CCL-210 cells in co-culture as compared to monoculture, which could point towards fibroblast signaling that stimulates fibroblast cell proliferation in cancer co-culture (Fig 10). These initial trends in gene expression may point to the importance of TIMPs in the changes in cell behavior observed in the studies presented here. However, further investigation to verify these results will be necessary in order to draw specific conclusions.
**Figure 10: Preliminary RT-qPCR data.** Graphs depict initial trends in expression of TGFβ (a), MMP2 (b), TIMP2 (c), MMP11 (d) for A549 cysts and CCL-210 cells alone and in co-culture, normalized to TATA-box binding protein using a ΔΔcq normalization method.

*note: data has not yet been obtained for MMP-11 for the CCL-210 cells.*
8. Supplemental Figures

Supplementary Fig. 1 Plots depict percent of nuclei positive for EdU at the two time points comparing healthy versus cancer epithelial cells, separated by monoculture and co-culture condition. Results are presented as means ± SEM of three biological replicates of each condition. ***p < 0.001

Supplementary Fig. 2 MMP activity on days 1 and 2 after encapsulation as measured by an MMP-sensitive fluorescent sensor peptide normalized to cell count for A549/CCL-210 co-cultures exposed to either 0.5% DMSO, 10 µM GM6001 or 100 µM GM6001 in the media.
Supplementary Fig. 3 Click-iT Plus EdU proliferation assay. Plot depicts percent of fibroblast nuclei positive for EdU on day 4 in monoculture and the A549/CCL-210 co-cultures, separated by gel and media type. The degradable bars refer to the original experiment in MMP-degradable gels with regular growth media. GM6001 was added at 10 µM, and the DMSO control media contained 0.05% DMSO. The nondegradable gels contained a peptide crosslinker insensitive to MMP cleavage. Results are presented as means ± SEM of three biological replicates of each condition. *p < 0.05, NS = not significant.

Supplementary Fig 4. Media used for 2D culture prior to 3D culture does not effect cell proliferation. Click-It Edu assays were performed on day 1 and day 4 of 2D culture. Plot depicts percent of nuclei positive for EdU. Results are presented as means ± SEM of three biological replicates of each condition.
Acknowledgements

There are not sufficient words for how grateful I am to the following individuals for making this work possible and helping me become the scientist I am today:

Foremost, I would like to thank Dr. Kristi Anseth for her mentorship, and her continuous support throughout this project and my education. You have been, and continue to be, a huge inspiration for me.

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Thank you to Esther Choi for a seemingly endless number of late nights working
through biological and experimental problems. Your love of science is contagious.

Finally, thank you to Terry, Tim and Avery Hall, and Jake Michael for their endless love and support throughout this process.

10. References


University of Colorado Boulder
Institutional Animal Care and Use Committee
APPLICATION TO USE VERTEBRATE ANIMALS
FOR RESEARCH OR TEACHING

Principal Investigator: Kristi Anseth
Department: Chemical Engineering
Phone: (303)-735-5336
Email: kristi.anseth@colorado.edu

Faculty Responsible for Project if PI is not a faculty member:
Phone:
Email:

Confirm pre-review by IACUC Departmental Representative with typed initials: ___SJB___

Project/Course Title: Isolation of lung epithelial cells from mice

Funding Source: Howard Hughes Medical Institute
Grant Number: N/A

Important: If you have a grant, contract, or proposal that will be associated with this animal protocol, please submit it along with the electronic Word version of the protocol via email to iacucoffice@colorado.edu

Project Start Date: 1/1/15
Project End Date: 1/1/18

This is a:

X New Application

Renewal Application. Previous Protocol Number: ____________

A. REQUIRED PROTOCOL SUMMARY (PI checks all that apply)
This protocol involves:

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APPLICATION CHECKLIST:

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<td>Protocol has been reviewed for scientific merit by an external organization (e.g. NIH), or the protocol was reviewed for scientific merit and signed by the Department Chair/designee/IACUC representative. Contact the IACUC office if you don’t know who should review your protocol for scientific merit.</td>
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<td>A version with the PI/faculty advisor’s signature has been sent to IACUC Office.</td>
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<td>Protocol has been pre-reviewed by the Veterinarian if pain category D or E procedures are involved. This pre-review should be simultaneous to the pre-review by the IACUC departmental representative. D or E categories of pain involve more than slight or momentary pain or distress to an animal.</td>
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<td>Protocol has been reviewed by the IACUC Representative for your Department (ask the representatives to initial the first page or email the IACUC Administrator indicating that they have reviewed the protocol). Send your protocol to your IACUC rep ONE WEEK before the protocol submission deadline for the upcoming IACUC Meeting. If you don’t know who your IACUC Representative is, contact <a href="mailto:iacucoffice@colorado.edu">iacucoffice@colorado.edu</a></td>
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For field studies, submit all applicable permits and field standard operating procedure.

If this protocol involves Radiation or Biosafety Hazards, those aspects need to be approved by the IBC or Radiation Safety Committee.

B. REVIEW OF SCIENTIFIC MERIT
This project has been reviewed for scientific/instructional merit by *(MUST be reviewed by at least one option below)*:

a. A federal agency (list): ____________

b. A non-governmental agency with peer review, excluding drug and device companies (specify and explain peer review process) ____________

c. The Department designee (initial here) ____ SJB ________
PRINCIPAL INVESTIGATOR CONTRACT

Animals Used for Research

I certify that the information in this application is true and, if applicable to the aforementioned funding source, that the protocol is essentially the same as found in the grant application or program/project.

Animals Used for Teaching

I certify that the information in this application is essentially the same as contained in the course outline and a copy of the laboratory exercises using animals is on file with the IACUC Office.

1. I certify that I have determined that the research proposed herein is not unnecessarily duplicative of previously reported research.
2. I certify that all individuals working on this proposal are participating in the institution’s occupational health and safety program.
3. I certify that the individuals listed herein are authorized to conduct procedures involving animals under this proposal, have attended the Basic IACUC training course at CU Boulder and have received or it is scheduled that they will receive further training to demonstrate proficiency in required procedures.
4. I certify that I have reviewed the pertinent scientific literature and the sources and/or databases and have found no valid alternative to any procedures described herein which may cause more than momentary pain or distress, whether it is relieved or not.
5. I certify that I will obtain approval from the IACUC before initiating any significant changes in this study.
6. I certify that I will notify the IACUC regarding any unexpected study results that impact the animals. Any unanticipated pain or distress, morbidity or mortality will be reported to the attending veterinarian and the IACUC (see statement below).
7. I certify that I am familiar with and will comply with all pertinent institutional, state, and federal rules and policies.

Pl's Name: Kristi Anseth Signature: Date: 11/19/2014

Advisor's Name: Signature: Date:

IACUC Adverse Event Statement:
The investigator will notify the IACUC (303-492-3411; 303-492-8187; UCB.Veterinarian@colorado.edu) of any unexpected results that adversely affect the welfare of the animals. The investigator will report any unanticipated pain or distress, morbidity or mortality to the attending veterinarian and the IACUC.

If there is an adverse event involving the health and/or safety of any research or animal handling personnel, the individual adversely harmed should report the event to their supervisor immediately. The supervisor should notify the Biosafety Officer ASAP at 303-492-8683 or at holly.gatemayer@colorado.edu. The person who is injured/ill should file a Worker’s Compensation Injury Report Form within 4 days of the work-related injury/exposure/illness. Sharps injuries must be reported with a special needle stick exposure form on the University Risk Management website. All injury reporting forms can be found at the URM’s website: https://www.cu.edu/content/fileclaim. For more information about Worker's Compensation, check out URM’s link here: https://www.cu.edu/articles/upload/WC049_DMP_PostG_11_09_01.pdf.
C. RATIONALE FOR USING ANIMALS
Summarize the objectives of this animal research or teaching project using non-technical language that a layperson (defined as an 8th grade science education) can understand. In lay terms, include the general methodologies used and the scientific or educational relevance of the project. Define acronyms the first time you use them. Do not use a grant abstract because it is too specific and not in lay language. Note: This is the only part of your protocol that could, if requested under the Freedom of Information Act (FOIA) or the Colorado Open Records Act (CORA) be made public, though there may be exceptions that would allow the abstract to be kept confidential. Please write your abstract with this in mind.

The goal for this project is to test the ability of synthetic materials to recreate the native environment of the lung in vitro. Specifically we will grow, within these materials, lung cells taken from mice and use this well-controlled model to study disease and developmental processes.

Current research on lung development and disease is typically done either in live animal studies or by growing lung cells as flat layers on plastic dishes or membranes. Live animals are complex systems with many uncontrollable variables, which makes in vitro studies attractive for investigating the influence of specific factors on cellular responses. However, flat cell cultures lack many of the physical cues present in lung tissue. Therefore, our objective is to create a 3D environment in which lung cells experience the same tissue structure found in the lung. Specifically, we will use a polymer-based material similar to that used in contact lenses (called a hydrogel) to entrap lung cells in the same hollow sphere shape found in the air sacs of lungs. With this model we can study the effect of various environmental cues on the lung cells, as well as grow them in close proximity to other cell types to better mimic their native state. We anticipate that with this 3D structure we will be able to predict what happens in vivo better than the previously studied flat cultures. This could be useful in understanding fundamental processes in lung development and disease and in developing potential therapeutics.

Our initial work in developing the materials for this model used a cell line derived from a lung tumor, but in order to be relevant to healthy lungs or the transition from healthy to diseased lungs, we need to use lung cells taken from healthy animals. Significant research has already been done with cells taken from mice (Kloxin, et. al., Integrative Biology, 2012) so in order to directly compare our results with those previous studies we are proposing to use adult male mice (FVB/NJ strain) for our cell source. We will order mice of the correct age (6-8 weeks old) from a vendor (Jackson Labs), euthanize them immediately upon receipt, and remove the lung tissue for use in cell culture on our hydrogels.
D. **SPECIFIC EXPERIMENTAL PROCEDURES INVOLVING ANIMALS:**

The purpose of this section of the protocol form is to understand the entire series of procedures that a single animal will go through on this application. Briefly explain the experimental design and specify all animal procedures. All procedures to be employed in the study must be described. This description should allow the IACUC to understand the experimental course of an animal from its entry into the experiment to the endpoint of the study. A flowchart may be an effective presentation of the planned procedure.

**Note:** Do not cut and paste procedures from the grant; organize the procedures for easy understanding. Spell out acronyms at least once. For renewals do not simply cut and paste previous protocol and addenda; only what is currently being conducted should be listed and reference to previous addenda should be removed, procedures reorganized and redundancies eliminated so the procedures will be easy to follow. Feel free to reference other portions of this protocol application if you have explained or justified a procedure in another part of this form, or provided details elsewhere.

Include the following specific information if applicable:

- **Animal identification methods** [e.g., ear tags, tattoos, collar, cage card, implant, etc.]
- **Methods of restraint** [e.g., restraint chairs, collars, vests, harnesses, slings, etc.]. Describe how animals are restrained for routine procedures like blood withdrawals. Prolonged restraint must be justified with appropriate oversight and monitoring to ensure it is minimally distressing. Describe any sedation, acclimation or training to be used. Include any signs of distress requiring removal from the study.
- **Blood withdrawals** [volume, frequency, withdrawal site, and methodology].
- **Radiation** [dosage and schedule].
- **Food or fluid restriction** If food, or fluid, or both food and fluid, will be restricted, describe method for assessing the health and wellbeing of the animals. [*Amount earned during testing and amount freely given must be recorded and assessed to assure proper nutrition.*] If you are seeking a departure from the recommendations of the Guide, provide a scientific justification.
- **Substances administered** [dose, frequency, route, duration, and volume]
- **Other procedures** [e.g., survival studies, tail biopsies].
- **Resultant effects**, if any, that the animals are expected to experience [e.g., pain or distress, ascites production, etc.].
- **Other potential stressors** [e.g., noxious stimuli, environmental stress]
- **Surgical procedures** [complete description starting from the pre-operative preparation to the recovery or euthanasia of the animal, including monitoring and documentation of anesthesia and post-operative procedures]
- **Method of euthanasia**
- **Specific endpoints** [time points, specific criteria]
- **Animals to be used for training personnel**

Animals to be used: adult male mice, FVB/NJ strain

No need for animal identification methods, because all are considered to be identical wild-type mice and they will be euthanized within 8 hours of receipt from the vendor.

No methods of restraint will be used.

No blood withdrawals will be done.

No radiation will be used.

No food or fluid restriction will be conducted.

No substances will be administered to animals other than CO2 for euthanasia.

Euthanasia is the only procedure to be performed.

Animals will experience no resultant effects.

No other potential stressors will be present.

No survival surgeries will be performed.
Euthanasia will be by CO2 asphyxiaition, followed by cervical dislocation and tissue dissection/lung cell isolation. Mice will be ordered at 6-8 weeks old and euthanized within 8 hours of receipt from vendor. The same mice will be used to train personnel in the tissue dissection technique and isolation of ATII cells.

** Provision in Case of Emergency  
If for any reason personnel are unable to euthanize mice within 8 hours of receipt from the vendor, animals will be moved to the OAR Holding Protocol for temporary housing.

**Project Scope**  
The Anseth Group of the Department of Chemical and Biological Engineering at the University of Colorado at Boulder is actively involved in designing new polymer-based hydrogel materials and techniques that promote long-term alveolar epithelial cell survival and function in 3D tissue structures relevant to the native lung. Current methods for in vitro culture of primary epithelial cells (e.g. monolayers) are limiting, as they do not mimic the spatial organization of alveolar tissue (e.g. hollow cysts surrounded by extracellular matrix (ECM) and other cell types such as fibroblasts and endothelial cells).

To test the efficacy of our newly designed materials, we plan to use primary alveolar epithelial cells isolated from mice. Immortal cell lines that are commercially available are inadequate because they are typically derived from tumors, and these genetic modifications allow them to rapidly proliferate in tissue culture conditions (normal alveolar epithelial cells have a very slow turnover rate) and their functional response is not representative of healthy alveoli. With proper biomaterial designs and fabrication, we expect that the isolated epithelial cells will form representative models of native alveoli in a well-controlled material system that can be used to test environmental signals on cellular response.

Two major lines of inquiry will be pursued under this protocol:
1. **Material-directed cell differentiation:** Material properties, such as binding epitopes (ECM peptides and full proteins) and hydrogel stiffness, will be varied to determine how these factors influence the differentiation of primary alveolar type 2 epithelial cells upon culture in vitro. Typically these cells differentiate into the type 1 phenotype. However, external cues have been shown to maintain the type 2 phenotype longer (Isakson, et al., In Vitro Cell. Dev. Biol.—Animal, 2002). With this knowledge, we can direct the dominant phenotype present in our model alveoli.
2. **In vitro model alveoli:** Hydrogels will be used to template and entrap single layers of primary epithelial cells in hollow cyst structures. These model alveoli will be used to test primary cell response to co-cultures with fibroblasts derived from healthy and pulmonary fibrosis cell lines. In addition, model alveoli will be exposed to biochemical signals from both the inside and the outside of the cyst to test primary cell response to these factors.

**Lung Dissection**  
Mice of the appropriate age (6-8 weeks) are ordered from a vendor and euthanized within 8 hours of receipt for tissue harvest. Mice are euthanized by CO2 asphyxiation and cervical dislocation. Then the surgery area of the animal is soaked with 70% ethanol. The chest is opened to expose heart lung block, and 0.25-1.0 mL of heparin 1000u/ml is injected into the left ventricle of the heart. The inferior vena cava is cut and 5-20mL of sterile saline is injected.
into right ventricle to clear the blood. Finally, the heart lung block is excised with a section of trachea attached and placed into 37°C phosphate-buffered saline solution.

Alveolar Type 2 Epithelial Cell Isolation
Lungs that did not flush well or look bad are discarded. Remaining lungs are trimmed away from heart, trachea and other tissue and finely minced with scissors or #22 scalpel blade. 50 ml collagenase digestion mixture is added to lung pieces, pipetted up and down, and transferred to a 250 ml glass bottle using a long cannula. The solution is incubated on rotator for 20 min at 37°C. Then, 10 ml trypsin is added and incubated on rotor for 20 min. A cannula is used to break up remaining lung pieces. Next, 50 ml trypsin inhibitor solution is added and mixed well. The lung mixture is passed through a 100µm cell strainer to remove large undigested lung sections, followed by filtration through a Nitex Filter 10um pore diameter. The filtered lung solution is transferred to 50mL tubes and centrifuged 8 min at 1300rpm. The supernatant is removed, leaving 5mL, and the tube is filled to 50ml with wash media. Cells are resuspended by vortex. Immune cells are separated from epithelial cells by adding cell suspension to tissue culture plates coated with IgG and incubating 1 hour at 37°C. Non-adherent cells are recovered and centrifuged at 1300 rpm for 8 min. Supernatant is removed and cells are resuspended in media of choice (usually DMEM/F12+10%FBS).
E. **LOCATION OF LIVE ANIMAL USE** (list all that are applicable for this protocol).

   Laboratory Room Number(s): Euthanasia and tissue extraction will take place in one of the procedure rooms within the vivarium, A1B71C and A1B71G.

   Animal Facility where animals are housed (not room number. Example: Ramaley Facility): Mice will not be housed. They will be euthanized within 8 hours of receipt from vendor.

   Animal Facility where procedures take place (not room number): Biofrontiers

   Location where surgery takes place (please provide room number): No recovery surgeries will be performed, only euthanasia.

   Additional applicable locations (i.e. satellite facilities): N/A

F. **PERSONNEL**

   The P.I. must document that each person is qualified to conduct the applicable procedures listed on this protocol or is being trained by qualified individuals in the lab.

   Please submit your training documentation with your protocol or renewal. There are two options and examples will be provided on request: 1) Individual training qualification document (Word); 2) Training document by lab (Excel).

   One of the two forms of training documentation (NOT BOTH) has been submitted for all personnel listed on this protocol, and this documentation is currently up-to-date.

   Yes [X] No

G. **SPECIFIC DETAILS ON ANIMAL USE AND CARE**

   **NOTE FOR RENEWALS ONLY:** When considering the number of animals needed for this renewal application, please carefully consider the animals that have been used in the previous three years. Be sure to justify the number of animals requested in the later sections of this form. If you need fewer animals for the renewal to achieve the same scientific aims, please request fewer animals.

1. **ANIMAL NUMBERS** (discuss species here, discuss specific strains in K4 below)

<table>
<thead>
<tr>
<th>Species (common name and scientific name)</th>
<th># Requested per year</th>
<th>Total Requested for Three years</th>
<th># On Hand at any time</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mice, <em>mus musculus</em></td>
<td>360</td>
<td>1080</td>
<td>0</td>
</tr>
</tbody>
</table>

2. **SOURCE OF ANIMALS** (check all that apply and briefly describe)

   □   Animals will be bred at CU Boulder

   [X] Animals will be ordered from vendors (list)*, or obtained from a collaborator (describe)*: Jackson Labs

   □   Animals will be collected (describe any permits that have been obtained, will be obtained)*:

   □   Other:

   *Animal transport and some biologically-derived products from an off-campus site may endanger the health of animal colonies on campus. In addition to notifying the IACUC by email to receive approval to order animals, such transport requires prior approval by the veterinarian. Prior to using custom antibodies in vivo, contact the veterinary staff to discuss appropriate biological monitoring for adventitial...
pathogens. Before receiving custom antibodies contact the IACUC office in order to ensure that the appropriate documentation is in place. Custom antibodies are produced using antigen(s) provided by or at the request of the investigator and not purchased off-the-shelf. An organization producing custom antibodies for an awardee must have or obtain an Assurance, or be included as a component of the awardee’s Assurance. In addition, the awardee must provide verification of project-specific IACUC approval for the production of the antibodies.

3. If animals are to be housed at CU Boulder, have you discussed arrangements for their housing with the applicable facility manager? Yes__________ No ______ X _______
   Mice will not be housed; they will be euthanized within 8 hours of receipt, and this has been discussed with the Biofrontiers facility manager.

4. Do you plan to remove animals from the vivarium/animal facility in which they are housed? If so, for how long will they be out of the vivarium? No.

5. If you answered question 4 above, do you plan on returning animals to their housing facility after procedures are completed? Note: before transporting animals to and from the animal facilities you must have approval from the animal facility manager to verify that the standard operating procedure is followed. In general, animals should be transported in cages that are within secondary containment.
   Yes__________ No________ Not applicable.

6. Will animals be held or housed in a satellite location/field research location for more than 24 hours on this protocol? (12 hours for USDA covered species)
   Note: if you answered yes to question 6, you must have an approved satellite application on file. If you don’t, go to the IACUC website and download the application next to SOP 18. Submit the completed application with your protocol form.
   Yes__________ No ______ X ______

7. Will you conduct procedures at another institution? If yes, please explain. Note: Ensure that you have prior approval before transporting animals and conducting work in a new location.
   Yes__________ No ______ X ______

8. Describe any enrichment you will provide your animals during the course of your research. The IACUC expects that all rodents (a social species) are group housed in addition to being provided a form of material enrichment unless there is a scientific or animal welfare reason for not doing so. Nestlets for mice are considered material enrichment. If animals will not be provided material enrichment, please provide scientific justification here: Mice will be euthanized within 8 hours of receipt from vendor.

9. Will animals be singly housed at any point during the course of this study?
   Yes__________ No________ N/A ______ X ______
   If yes, please indicate which animals in the study will be singly housed and provide a justification for this practice here:

10. LIST personnel responsible for daily animal care (in accordance with the Guide animals must be monitored at least daily including weekends and holidays). Please note that investigator provided husbandry and monitoring requires a special care form: Margaret Isenhart and Michael Antczak provide daily animal care in the Biofrontiers vivarium. However, these mice will not be housed. They will be euthanized within 8 hours of receipt from the vendor.
H. ANIMAL PROCEDURE DETAILS

1. List the names of personnel conducting the non-surgical procedures:
   No procedures other than euthanasia will be performed.

2. Summarize OTHER DRUGS/TREATMENTS USED ON PROTOCOL (e.g. E. Coli, other biological solutions, drugs not used for anesthesia, analgesia, euthanasia) You may list the ranges of doses and ranges of frequencies if there are multiple combinations on this protocol.

<table>
<thead>
<tr>
<th>Species</th>
<th>Drug(s)</th>
<th>Dose</th>
<th>Frequency of Administration</th>
<th>Duration of Treatment</th>
<th>Route of Administration</th>
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<tbody>
<tr>
<td>a.</td>
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<tr>
<td>b.</td>
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<td>c.</td>
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3. Will surgery be done? Yes ______ No ______ x

4. Will more than one major survival surgery be performed on a single animal while on this protocol? Yes ______ No ______ x
   If yes, why must you perform two or more major survival surgical procedures on a single animal:

5. Will this project involve recovery from anesthesia? Yes _____ No _____ x

6. Personnel conducting the surgical procedures (list out the names of specific personnel): No surgeries will be performed, prior to euthanasia.

7. Describe the pre-operative preparation procedures for the animals: No surgeries will be performed, prior to euthanasia.

8. Describe the sterilization methods and aseptic procedures that will be used for survival surgeries: No surgeries will be performed, prior to euthanasia.

9. How frequently will the animal be observed during surgery? No surgeries will be performed, prior to euthanasia.
10. How frequently will the animal be observed during immediate surgical recovery? **No surgeries will be performed, prior to euthanasia.**

11. If you plan to use anesthetics, analgesics or tranquilizers to alleviate pain or distress, please supply the following*: If analgesics are not to be used to alleviate pain, provide detailed justification in Item K1 and check Pain Category E. Please check our website for a helpful **drug formulary** created by CU Denver Anschutz.

<table>
<thead>
<tr>
<th>Species</th>
<th>Drug(s)</th>
<th>Dose</th>
<th>Frequency of Administration</th>
<th>Duration of Treatment</th>
<th>Route of Administration</th>
</tr>
</thead>
<tbody>
<tr>
<td>a.</td>
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</table>

12. Outline the type of postsurgical care that will be provided and its frequency/duration. Include the frequency of monitoring. **No surgeries will be performed, prior to euthanasia.**

13. Who is responsible for monitoring post-operative care or post-procedural care (e.g. surgery, or injection of tumor cells)? **No surgeries will be performed, prior to euthanasia.**

14. If anesthetic gases are going to be used, please describe how the gases will be scavenged: **No anesthetic gasses will be used. CO2 (provided by the vivarium) will be utilized for euthanasia.**

15. Are you using any **non-pharmaceutical grade drugs and compounds** in this protocol**?**

A pharmaceutical grade compound is a drug, biologic, or reagent that is approved by the Food and Drug Administration (FDA) or for which a chemical purity standard has been established by the **United States Pharmacopeia-National Formulary (USP-NF)** or the **British Pharmacopeia (BP)**. According to guidance from the FDA, “pharmaceutical secondary standards” are acceptable for use in clinical animal studies if obtained from a reputable source and comply with compendia standards. From OLAW’s FAQ June 18 2013

Yes______ No ______

16. If yes to #15 above, please provide a scientific justification for the use of these items and provide information as to why you are not able to use available pharmaceutical grade alternatives.
17. If yes to #15 above, please list all the non-pharmaceutical grade compounds to be used and explain how these compounds will be prepared so as to compensate for not being pharmaceutical grade, including sterility. Justification based on prior experience with the compound is also useful (e.g. no previous adverse consequences of such agents). * OLAW and USDA agree that pharmaceutical-grade chemicals and other substances, when available, must be used to avoid toxicity or side effects that may threaten the health and welfare of vertebrate animals and/or interfere with the interpretation of research results. However, it is frequently necessary to use investigational compounds, veterinarian- or pharmacy-compounded drugs, and/or Schedule I controlled substances to meet scientific and research goals. The IACUC is responsible for evaluating the potential adverse consequences of such agents when used for research. In making its evaluation, the IACUC may consider factors including, for example: grade, purity, sterility, acid-base balance, pyrogenicity, osmolality, stability, site and route of administration, compatibility of components, side effects and adverse reactions, storage, and pharmacokinetics.
I. METHOD OF EUTHANASIA and ENDPOINTS

Euthanasia of animals. For a discussion of acceptable methods of euthanasia, see the AVMA Guidelines for the Euthanasia of Animals: 2013 Edition. Also, see SOP 6 Euthanasia on the IACUC website. A secondary physical method of euthanasia after carbon dioxide or an injectable euthanasia solution is required for rodents.

<table>
<thead>
<tr>
<th>Species and Age</th>
<th>Drug/Method</th>
<th>Dose</th>
<th>Route of Administration</th>
</tr>
</thead>
<tbody>
<tr>
<td>a. mice, adult</td>
<td>CO2 asphyxiation</td>
<td></td>
<td>Inhalation</td>
</tr>
<tr>
<td>b. mice, adult</td>
<td>Cervical dislocation</td>
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<td>c.</td>
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</table>

1. If your methods of euthanasia differ from the AVMA Guidelines 2013 Edition, (i.e., not using tranquilizers or anesthetics before decapitation or cervical dislocation) describe why such a method is scientifically required here. Note! The use of cervical dislocation as the sole method of euthanasia is not a recommended method of euthanasia by the 2013 AVMA Guidelines. If you wish to use cervical dislocation alone, please scientifically justify thoroughly here: CO2 asphyxiation will be used prior to cervical dislocation and lung dissection.

2. Personnel that are trained and will perform euthanasia (list individual names): Katherine Lewis, Emi Kiyotake

3. Scientific Endpoints. Please describe at what point animals will be euthanized as part of this project. If the final disposition is not euthanasia, please describe here (e.g. animals will be used for training, they will be adopted, etc.): 6-8 weeks old, within 8 hours of receipt from vendor

4. Humane Endpoints. Please describe all potential adverse events and expected outcomes on this protocol that could compromise the health of the animal (i.e. anesthetic death, ulcerated tumors, significant loss of body weight, severe skin lesions, etc). If it is expected that animals will become moribund as a result of this study, please describe very specifically the signs you will watch for, and describe the point at which an animal will be euthanized to alleviate their pain/distress prior to the experimental endpoints: Animals will be euthanized upon receipt from vendor. They will be monitored during CO2 asphyxiation, and we will wait 2-3 minutes after breathing has stopped and death is confirmed by toe-pinches before proceeding with cervical dislocation and tissue dissection.

5. For illness, injury, or abnormal behavior how will these outcomes be managed (e.g. frequent monitoring, consultation with the veterinarian, euthanasia if a tumor reaches a certain size, etc.). Note: The Institutional Veterinarian should be notified of any unexpected deaths, illness, distress, or other deviations from normal in animals.

   At time of receipt from vendor animals will be visually assessed for health. If an animal if found to be ill we will consult with the veterinarian or veterinarian technician

6. Are you using a controlled substance? Yes _______ No _______ X _______

   If no, skip to section J below.

   a. Do you have a DEA license? Yes _______ No _______
   b. Who will purchase these drugs (name):

All investigators must maintain records of the drugs, store them as required by the DEA, and conduct an inventory once every two years as required by CU Boulder Policy.
J. PAIN CATEGORIES, PAIN AND DISTRESS IN ANIMALS
The answers to the following questions are necessary for the Institutional Animal Care and Use Committee to fulfill its obligations under the Public Health Service Policy. Investigators and course directors are requested to categorize their use of vertebrate animals based on the discomfort or pain involved. Consideration should be given to methods that result in a lesser degree of unavoidable pain or discomfort, and the use of the smallest number of animals consistent with meeting the scientific or educational objectives. It is also necessary to provide assurance that animals and personnel working with animals are not unduly exposed to a hazardous environment.

Please check ALL of the discomfort/pain categories that apply to your research project and briefly describe the procedure under that category.

CATEGORY B: Animals being bred, conditioned, or held for use in teaching, testing, experiments, research, or surgery but not yet used for such purposes.
Definition: Breeding Colony Protocols or Holding Protocols

CATEGORY C
Definition: Procedures conducted involving no pain or only slight or momentary pain and distress no greater than an injection and no use of pain relieving drugs. [i.e. injections, tail snips for genotyping, routine handling, innocuous behavioral observations]
Briefly list procedures in category C: euthanasia by CO2 asphyxiation and cervical dislocation

CATEGORY D
Definition: Experiments, teaching, research, experiments, or tests conducted involving more than a slight or momentary pain or distress to the animals for which appropriate anesthetic, analgesic, or tranquilizing drugs will be used.
Briefly list procedures in category D:

CATEGORY E
Definition: Procedures conducted involving more than a slight or momentary pain or distress to the animals for which the use of appropriate anesthetic, analgesic, or tranquilizing drugs cannot be used because they will affect the procedures, results, or interpretation of the teaching, research, experiments, surgery, or tests. Examples are: prolonged restraint, food and water deprivation, toxicology experiments, etc.

Category E procedures must be thoroughly described in I, scientifically justified in K9, and steps to minimize the pain including specific criteria that will be used to end the study, provide analgesia, or euthanize the animals so as to limit the pain or distress must be indicated in K1.
Briefly list procedures in category E:

For more specific examples of each USDA pain category, please refer to: USDA Pain Categories
K. JUDICIOUS USE OF ANIMALS
All the questions below are applicable to ALL protocols. Do not leave one blank.

1. **Refinement**: List measures you will take to ensure that pain, distress, discomfort and injury will be limited to that which is unavoidable in the conduct of scientifically sound research. This is a standard question on grant applications involving animals. We require this answer on this protocol regardless of whether this project is federally funded. If you have already provided this answer indicate where in the protocol it is located.

   No procedures will be performed on living animals other than the initial euthanasia.

2. **Replacement**: Could mathematical models, computer simulation or in vitro biological systems be used as alternatives to the use of animals in this project? Explain. **For Guidance on searches for alternatives go to this hyperlink on the DU Library website**

   The complexity of the processes being studied cannot be duplicated or modeled in simpler systems. Extensive work with cell lines has been done to develop the materials and the templating procedure. Additionally, immortal cell lines that are commercially available are inadequate because they are derived from tumors, and the genetic modifications allow them to rapidly proliferate in tissue culture conditions (normal alveolar epithelial cells have a very slow turnover rate) and their functional response is not representative of healthy alveoli.

3. Justify the use of the animal species listed. Describe the biological characteristics of the animal that are essential to the project. This is, phylogenetically, the lowest species that provides adequate size, tissue or anatomy for the proposed study. It provides a particularly good model for duplicating the human condition. Previous studies using this species formed the background for this project. A large database exists, allowing comparison with previous data.

4. **Reduction**: Justify the number of animal used. Describe the size and number of experimental groups and the number of animals needed for procedure development. "Whenever possible, the number of animals and experimental group sizes should be statistically justified" (page 25 of the Guide). If you can do a power analysis or some other statistical method to justify animal numbers, do so here. Include the parameters you used. If there are a lot of experiments/animals used on the protocol it is helpful to provide a summary table. Regardless, the total numbers of animals should match those in the table in section G1.

   The numbers of animals were determined by the number of ATII cells needed for cell differentiation and model alveoli studies. We performed a power analysis assuming a population variance based on cellular response from previous studies. We estimate that three replicates per condition are required to observe the expected effect size between control and experimental groups. Our collaborators have isolated alveolar type 2 epithelial cells from 6-8 week old FVB/NJ mice for several years, and have determined empirically that one mouse yields about 1-2 million cells (Dr. Vivek Balasubramaniam, personal correspondence). For each experiment, roughly 20-40 hydrogel conditions will be used, including 3 statistical replicates, on average testing 10 hydrogel and model alveoli conditions per experiment. Each hydrogel requires a seeding density of 0.5 million cells per hydrogel. Therefore, 10-20 mice are required per experiment. Over the course of this project we expect to perform about 3 experiments every 2 months, or 18 experiments to test approximately 180 conditions per year. That means that the maximum mice needed is 360 mice per year (20 mice/experiment x 18 experiments/year), and 1080 mice over 3 years. However, if more than 1 million cells can be isolated per mouse, or fewer conditions are tested, then a lower number of animals will suffice.

   It should be noted that extensive work is done with cell lines to characterize and develop our material systems before using primary alveolar epithelial cells. Because of this, we are able
to dramatically reduce the number of animals needed to develop our materials. Validation of cell line-based experiments with primary cells, however, is a critical proof of concept step that significantly increases the medical relevance of our work.

5. Are any State or Federal permits required for this protocol (e.g., permits to obtain wild animals, importation permits for animal tissue, etc.)?
   X No  ___ Yes
   If "Yes," submit copies of all permits with the application.
   If "Yes," submit your standard operating procedure for the field that includes safety procedures for personnel and decontamination of study equipment between study sites, etc.

6. Provide written assurance that the proposed activities do not unnecessarily duplicate previous experiments. USDA and PHS Policy require that the investigator make a reasonable good faith effort in determining that a proposed experiment is not unnecessarily duplicative. For this assurance, you may choose to cite information from the introduction, significance or bibliographic sections of your grant proposal or any other material that may be relevant to this question.

   In planning this experiment, I have reviewed the relevant literature. Based on the available literature, I certify that the activities involving animals described in this protocol do not unnecessarily duplicate previous research. There is still much that is unknown about alveolar epithelial cells (Guillot, et. al., The International Journal of Biochemistry & Cell Biology, 2013), and 3D in vitro studies with primary cells are few and have only been done in less-defined systems like Matrigel and collagen (Yu, et. al., Molecular Biology of the Cell, 2007).

7. For projects that may cause more than momentary or slight pain or distress to the animals involved (Pain Categories D, or E), provide a written narrative describing the methods and sources (e.g., biological abstracts, Index Medicus, Current Research Information (CRIS), Animal Welfare Information Center, etc.) used to determine that less distressful alternatives were not available. Please be sure to include the search strategy used (i.e. the combination of search terms) and results to the search. If this protocol has pain categories of only B or C, state that. For Guidance on searches go to this hyperlink on the DU Library website.

   Pain category C only

8. For projects that may cause more than momentary or slight pain or distress to the animals (Categories D or E), consult with the veterinarian about how pain and distress can be effectively managed. Briefly document the major points of discussion during the consultation with the Institutional veterinarian. If this protocol has pain categories of only B or C, state that.

   Pain category C only

9. Scientifically justify pain/distress category E research here:

   Pain category C only
L. Additional Departures from the 8th Edition of the *Guide for the Care and Use of Laboratory Animals*

1. Does this protocol require >2 hours food or fluid restriction for animals? □ X No □ Yes

2. Will animals on this protocol be subjected to temperature or humidity conditions that differ from those provided in the animal housing facilities on campus? □ X No □ Yes

3. Animal enclosures and the objects within them (water bottles, sipper tubes, wire tops) are sanitized every one or two weeks, depending on the animal facility on this campus. Will you require a deviation from this policy for any structure that is part of the animal’s housing arrangement? (running wheels, enrichment objects, etc.) □ X No □ Yes

4. Will the animals described in this protocol be subjected to any deviation from the standard 12 hours light, 12 hours dark diurnal light cycle? □ X No □ Yes

5. Will physical restraint be utilized on this protocol for any animal, for duration longer than 15 minutes at a time? (physical restraint does not include routine restraint for animal handling, and it is assumed that handling restraint would be of a shorter duration) □ X No □ Yes

   If physical restraint will be for longer than 15 minutes, please describe the following immediately below question 7 or reference another section of your protocol:
   
   a. Describe specific criteria you will use to exclude an animal from a restraint regiment if an animal fails to adapt.

   b. Describe the training you will provide to the animal in order to adapt to the restraint paradigm and the personnel. Positive reinforcement should be used as a component of training if possible.

   c. Describe the intervals at which you will observe the animal while they are undergoing the physical restraint paradigm.

6. Does this protocol require sleep deprivation for any animal? □ X No □ Yes

7. If you have checked “YES” for any of the items 1-6 above, please thoroughly justify immediately below, or reference another section of the protocol where you have provided this justification:
# M. ANIMAL SAFETY AND PERSONNEL SAFETY SECTION

****Please ensure that all personnel in this protocol have taken the required IACUC Basic Training and are enrolled in the Occupational Health Program at CU Boulder. For information regarding this step, please see the [CU Boulder IACUC Training webpage](https://icuc.colorado.edu/).****

<table>
<thead>
<tr>
<th>1. Hazardous Waste:</th>
<th>Yes</th>
<th>No</th>
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<tbody>
<tr>
<td>Will you or anyone in your lab be generating, handling, or managing hazardous waste?</td>
<td>X</td>
<td></td>
</tr>
<tr>
<td>Will you or anyone in your lab be treating research animals with chemotherapeutics as a procedure on this animal use protocol?</td>
<td>X</td>
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<tr>
<th>2. Institutional Biosafety Committee (IBC) Application, Bloodborne Pathogen Training, and Biological Material Shipping Training:</th>
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<tbody>
<tr>
<td>Does your research involve the use of any biological agents, infected or potentially infected animals or their tissues/bodily fluids (including field work), recombinant or synthetic nucleic acid molecules, Select Agents and Toxins, or work with human blood, bodily fluids, tissues, or cells in culture?</td>
<td>X</td>
</tr>
<tr>
<td>If Yes, has an Institutional Biosafety Committee (IBC) Application been submitted and approved? If No, please contact EH&amp;S at 303-492-6025</td>
<td></td>
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<tr>
<td>If yes, please list date of approval:</td>
<td></td>
</tr>
<tr>
<td>Does your research involve the shipping or receipt of potentially infectious materials from humans or non-human animals, human blood, blood products, reagents derived from blood, human specimens such as tissue, saliva, sputum, etc?</td>
<td>X</td>
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<tr>
<th>3. Use of Human Subjects or Human Tissues:</th>
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<tbody>
<tr>
<td>Does your research involve collection, receipt, or use of human specimens?</td>
</tr>
<tr>
<td>If Yes, has the project been reviewed and approved by the Institutional Review Board?*</td>
</tr>
<tr>
<td>*If No, please contact the Institutional Review Board at 303-735-3702; <a href="http://humanresearch.colorado.edu/">http://humanresearch.colorado.edu/</a></td>
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<th>4. Radioactive Materials:</th>
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<tr>
<td>Will radioactive materials be used as part of the research listed in this Application?</td>
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<tr>
<td>If yes, are you licensed for the possession and use of each radioisotope?</td>
</tr>
<tr>
<td>*If No, please contact EH&amp;S-Health Physics at 303-492-6523.</td>
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<th>5. X-ray Machines:</th>
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<tr>
<td>Will X-ray Machines be used as part of the research listed in this Application?</td>
</tr>
<tr>
<td>If yes, are you licensed for the possession and use of each machine?</td>
</tr>
<tr>
<td>*If No, please contact EH&amp;S-Health Physics at 303-492-6523.</td>
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<th>6. Export Control:</th>
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<tr>
<td>Will the project include export or transport of material outside the US?</td>
</tr>
<tr>
<td>Are you working with foreign nationals, institutions, or students?</td>
</tr>
<tr>
<td>If you answered yes to either question in “6. Export Control” contact Linda Morris at 303-492-2889 or <a href="mailto:linda.morris@colorado.edu">linda.morris@colorado.edu</a></td>
</tr>
<tr>
<td><a href="http://www.colorado.edu/VResearch/integrity/exportcontrols/index.html">http://www.colorado.edu/VResearch/integrity/exportcontrols/index.html</a></td>
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<th>7. Conflict of Interest:</th>
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<tbody>
<tr>
<td>All faculty, PRAs and anyone who is responsible for designing, conducting or reporting research is required to disclose external professional activities on an annual basis (via the DEPA). If your research is funded by NIH or DHHS (PHS) you and your research team are required to complete CU Boulder’s COI training, initially and then every 4 years. Training information is populated in the DEPA system. <a href="http://www.colorado.edu/vcr/coi">http://www.colorado.edu/vcr/coi</a></td>
</tr>
<tr>
<td>Have you and your research team received DEPA approval for the current year? If No, please submit DEPAs:</td>
</tr>
<tr>
<td><a href="http://www.colorado.edu/vcr/coi">http://www.colorado.edu/vcr/coi</a> or contact <a href="mailto:pam.rosse@colorado.edu">pam.rosse@colorado.edu</a></td>
</tr>
<tr>
<td>X</td>
</tr>
<tr>
<td>Is any of your research funded by NIH or DHHS(PHS)? If yes, please answer the next question.</td>
</tr>
<tr>
<td>Have you and your team completed the required NIH/DHHS(PHS) COI training? If No, please complete training.</td>
</tr>
<tr>
<td><a href="http://www.colorado.edu/vcr/coi">http://www.colorado.edu/vcr/coi</a></td>
</tr>
</tbody>
</table>
N. SAFETY REVIEW

It is ultimately the principal investigator’s responsibility to ensure all lab and animal husbandry personnel are made thoroughly aware of the potential hazards of any chemical, biological, or radiological materials, hazardous gases (i.e. anesthetics) or procedures being used in their research or laboratories. If you are unsure of the risk involved, contact Environmental Health and Safety (EH&S) at 492-6025 for more information.

1. Will the use of any of the chemical, biological, or radiological materials, hazardous gases (i.e. anesthetics) or procedures listed in this application pose a direct hazard to health or safety of the animal colony or personnel working with animals?  
   No.

2. Describe the practices and procedures required for the safe handling and disposal of contaminated animals and material associated with this study. Include a description of the PPE (personal protective equipment) your lab will utilize at all times. Also describe methods for removal of radioactive waste and, if applicable, the monitoring of radioactivity:
   In the vivarium, goggles, lab coat, gloves, hairnets, shoe covers, facemasks, and scrubs will be worn to protect lab personnel at all times. After tissue dissection, animal carcasses will be transferred to resealable plastic bags, frozen at -20°C, and disposed of by animal facility personnel.

3. Additional safety considerations: None.

This protocol is certified approved by at least one of the individuals below:

[Signature]  12/30/2014
Chair, Institutional Animal Care and Use Committee  Date

[Signature]  D.V.M.
Director, Department of Animal Resources  Date

IACUC Administrator  Date