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Transcriptional Responses to Altitude-Induced Hypoxia in Bronchial Epithelium, Broncho-alveolar Cells, and Blood Differ Between Smokers and Nonsmokers.

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16. Abstract

The hypoxic environment of an aircraft cabin may exacerbate underlying health conditions related to respiratory ailments, including those induced by smoking. In order to assay the differences between smokers and nonsmokers during flight, we analyzed the gene expression profiles of bronchial epithelium, alveolar cells, and blood in smokers and nonsmokers before, during, and after exposure in a hypobaric chamber to an 8,000-ft cabin pressurization. Unique transcriptional changes were observed between smokers and nonsmokers exposed to flight conditions. Smoker bronchial epithelial cells exhibited inhibition of the hypoxic response and activation of cellular death pathways compared to nonsmokers. Smoker alveolar cells displayed a mixed hypoxic regulatory profile and activation of cellular recruitment and inflammation pathways. Smoker blood displayed very little difference to that of nonsmokers, but presented a restricted hypoxic response. In summary, smokers exhibit an inhibition of the hypoxic response compared with nonsmokers during exposure to flight conditions, resulting in alternate cellular responses that may mitigate the inhibition of the hypoxic response.

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Introduction

While at altitude, aircraft passengers and crew are exposed to mildly hypoxic environments wherein a passenger aircraft cabin maintains a pressure of approximately 565 mmHg, or the equivalent of 8,000 ft (14 Code of Federal Regulations §25.841, Muhm et al. 2007). Systemic hypoxia is experienced upon exposure to such low-oxygen or high-altitude environments, initiating a number of countering physiological and molecular responses (Peacock 1998, Jia et al. 2016).

The primary initial physiological responses to hypoxia are hyperventilation and tachycardia, initiated to counter decreasing blood oxygen saturation (SaO_2) caused by a reduction in oxyhemoglobin due to decreased oxygen availability (Nesthus et al. 1997, Sarkar et al. 2003, Samuels 2004, reviewed by Burki and Tetenta 2013). Secondary physiological effects of acute hypoxia include pulmonary hypertension induced by pulmonary vasoconstriction, increased cranial blood flow, and inflammation, and are accompanied by symptoms including headache, nausea, lethargy, weakness, and insomnia (Peacock 1998, Samuels 2004). These effects, when clinically evident, are collectively referred to as "Acute Mountain Sickness" (AMS) due to the common occurrence of these hypoxic symptoms during rapid ascents to elevations exceeding 7-8,000 feet (Peacock 1998, Samuels et al. 2004, Wu et al. 2012, Yan et al. 2015).

In addition to physiological effects, acute and chronic hypoxia also measurably alter gene expression. In vivo, tissues subsist at sub-atmospheric oxygen concentrations ranging from 14.5% in alveolar cells to 3.4 % in brain tissue, with a typical range of 3.4% to 6.8% for the majority of tissues (reviewed by McKeown 2014). Tumor tissues, due to their isolation from the vasculature and rapid growth, experience increased hypoxia, often < 1% oxygen (Muz et al. 2015, Eales et al. 2016). In tumor cells and other aberrantly-hypoxic tissues, the hypoxic response is typified by the expression and induction of a number of master regulatory genes, most commonly *HIF1a*, Semenza 1992), *MAPκ* (Richard et al. 1999), *ERK1/2* (Minet et al. 2000), *P38* (Huxtable et al. 2015), *mTOR* (Brugarolas et al. 2004), *NFκB* (Royds et al. 1998), *ATM* (Olcina et al. 2014), *VEGF* (Morfoisse et al. 2015), and *PI3k* (Kilic-Eren et al. 2013). The molecular hypoxia response is focused on activating HIF1a protein, a transcription factor which is hydroxylated by PHD and FIH-1 oxygen-sensing proteins, leading to ubiquitination and rapid degradation at normoxia (reviewed by Fandry et al. 2006, Muz et al. 2015). During hypoxic conditions, HIF1a hydroxylation ceases, and HIF1a is transported to the nucleus where it

heterodimerizes with the ubiquitously-expressed HIF1b to function as an activating transcription factor, triggering activation of downstream hypoxic responses including inflammation (Fandrey et al. 2006, Palazon et al. 2015), increased vascularization, and angiogenesis (Semenza et al. 2011).

The hypoxic response during altitude-induced hypoxia is considerably milder than that of the tumor microenvironment, but mild hypoxia is sufficient to induce a hypoxic response. Live-subject studies of hypoxia in rodents found upregulation of genes involved in vascular remodeling, and pulmonary hypertension, as well as the HIF1a targets *ADM*, *VEGF*, *HK2*, *PDK1*, *BNIP3*, *DUSP1*, and others (Fandrey et al. 2006, Wu et al. 2008, Trollman et al. 2010, Eales et al. 2016). Studies of Acute Mountain Sickness, a condition on the same altitude exposure spectrum as aviation-induced mild hypoxia, found similar transcriptomic changes, including an increase in the angiogenic *ANGPTL4*, *IL17F*, *IL10*, *CCL8*, and additional genes invoking angiogenic, inflammatory, and immune responses in hypoxic individuals (Goodin et al. 2013, Liu et al. 2017).

Smoking is a potential amplifier of the hypoxic response. Smokers display elevated carboxyhemoglobin levels due to CO inhalation and indications of chronic tissue hypoxia (Sagone et al. 1973), thus smokers are subject to chronic mild hypoxia in comparison with nonsmokers at ground level. Studies of smokers transported to high altitudes revealed a lower incidence of Acute Mountain Sickness in comparison with nonsmokers, perhaps due to prior existence of a hypoxic state in smokers, but a poorer long-term adaptation prognosis (Wu et al. 2012). Further, cigarette smoke is known to induce HIF1A protein accumulation under normoxic conditions, with corresponding increases in VEGF and REDD1 (Daijo et al. 2016).

In order to assay the extent of the hypoxic effect at cruising altitude on active smokers, we examined the physiological and transcriptional responses of smoking and nonsmoking groups at both flight and ground conditions, and analyzed that data to determine the presence of significant differences between each group. Because smokers may be at increased risk of hypoxia during flight, this study was designed to improve understanding of this subpopulation at cabin altitude. In particular, we wished to understand whether identical biomarkers may be used to detect mild hypoxia in smokers and nonsmokers.

Materials and Methods

Subject Recruitment and Study Design

All studies were performed under a protocol approved by the institutional review boards (IRB) of the University of Oklahoma Health Sciences Center, the Oklahoma City Veterans Administration Medical Center, and the Federal Aviation Administration. Human volunteer research subjects each granted their informed consent to participate in the study under the

auspices of the previously-mentioned approved IRB protocol. Subjects were selected based upon the absence of active or prior lung disease, normal pulmonary function test results, age between 25 yrs and 50 yrs, and absence of prior diagnosis, signs, or symptoms of respiratory or cardiac disease. Nonsmokers were defined as individuals who completely abstained from tobacco use, smokers were defined as individuals smoking between 5 and 15 cigarettes daily. Subjects were matched for age and gender between the smoking and nonsmoking groups.

The selected volunteer subjects participated in a randomized crossover study comparing male and female smokers and nonsmokers at both a normoxic "ground" phase (720 mmHg, 1,400 ft. equivalent) and mildly hypoxic "flight" phase (563 mmHg, 8,000 ft. equivalent) simulated altitudes in a hypobaric chamber situated at 1,200 ft. above sea level at the Federal Aviation Administration's (FAA) Civil Aerospace Medical Institute (CAMI), in Oklahoma City, OK, USA (Figure 1). Atmospheric pressure within the hypobaric chamber during the ground phase was maintained at an altitude equivalent of 1,400 feet to prevent participants from determining which simulation was actually the "flight" (F) or "ground" (G) part of the experiment. Each exposure lasted for 5.5 h, including 5 h at flight or ground altitudes, and an additional 0.5 h combined for ascent and descent, approximately the equivalent of a coast-to-coast U.S. domestic flight. Blood samples were collected at 4 timepoints, one before ascent (hour 0), two during the flight/ground exposure (1 h and 5 h following initial ascent), and one post-exposure (hour 6.5, approximately 1.5 h after exiting the chamber). At the final timepoint (hour 6.5), bronchoscopies were performed on each subject to obtain lung airway cells by brushing (Human Bronchial Epithelial Cells, HBEC), and alveolar cells by broncho-alveolar lavage (BAL cells). Each treatment phase was separated by a minimum of a 30-day "wash-out" period to eliminate residual effects of either treatment.

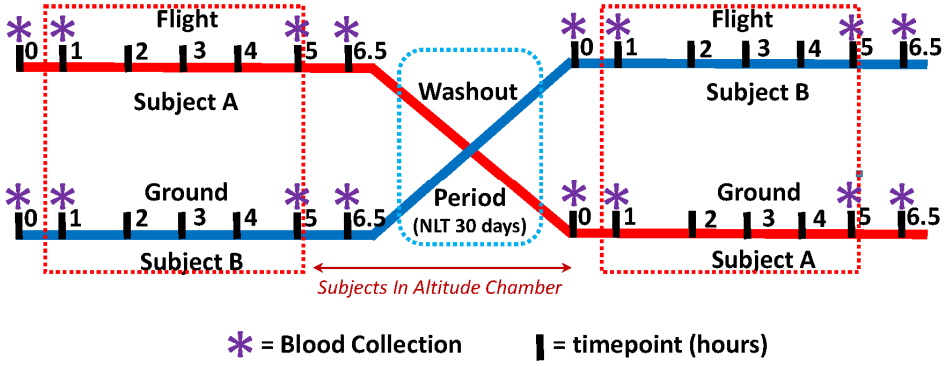


Figure 1. Crossover study designed to examine differences between smokers and nonsmokers during simulated flight. Each subject was exposed to both flight and ground conditions, separated by a washout period of no less than (NLT) 30 days. Subjects spent 5.5 hours in a hypobaric chamber for each study phase. Blood samples were taken prior to chamber entry (hour 0), after 1h and 5h within the chamber, and after exiting the chamber (hour 6.5h). Timepoints spent at flight or ground conditions within the hypobaric chamber are indicated by enclosure within red dashed boxes.

Determination of Physiological Responses at Flight and Ground

To assess immediate physiological responses during the study, pulse rate, blood oxygen saturation (SaO_2), and exhaled gas measurements (exhaled N_2, O_2, CO_2, and H_2O) were assessed. Blood oxygen saturation was measured at the fingertip by standard pulse oximetry devices and exhaled gas partial pressures were measured by subjects blowing into a party horn connected to a Perkin Elmer MGA-1100 gas chromatograph while subjects were in the hypobaric chamber during both the flight and ground sessions (Figure 2). Blood was drawn prior to entry into the hypobaric chamber (hour 0) and during flight/ground exposure at hours 1 and 5. Physiological measures (SaO_2, exhaled N_2, O_2, CO_2, and H_2O, and heart rate measurements) were taken prior to chamber entry (hour 0) and then approximately hourly during the chamber session (hours 1, 2, 3, 4, and 5). Additionally, blood, alveolar cells, and bronchial epithelial cells were obtained approximately one hour post-exposure (hour 6.5) for use in gene expression analysis.

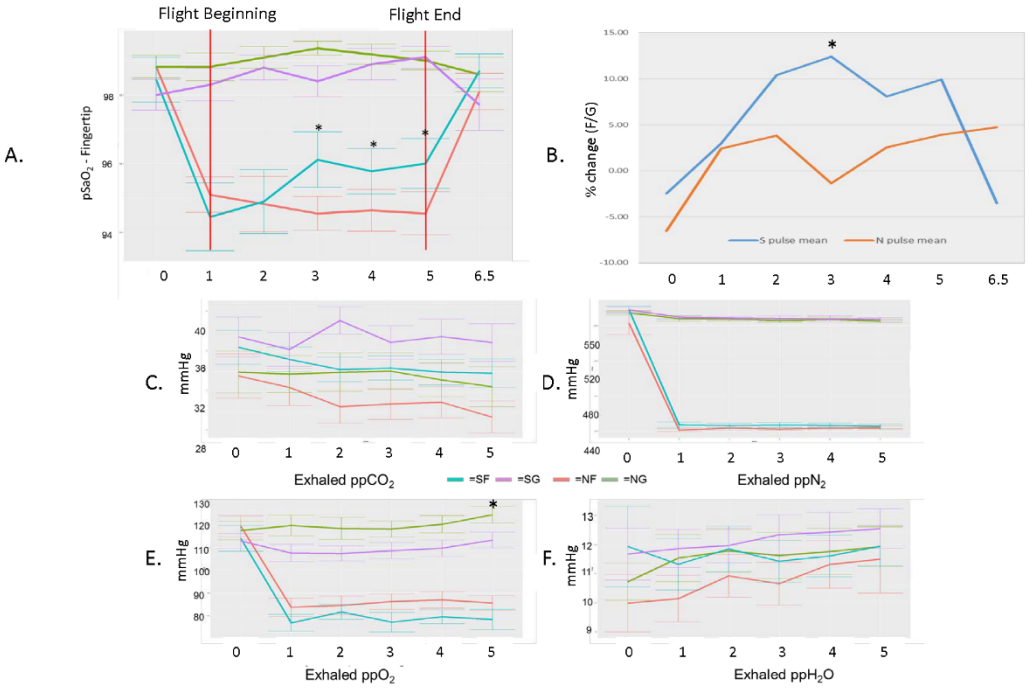


Figure 2. Blood oxygen saturation (finger) and exhaled gas measurements (as determined by gas chromatograph) over the course of the ground or flight altitude treatment. A. SaO_2 measurements by group. Timepoints represent hours before and after initial ascent. Points 0 through 5 are the times at flight or ground, and time 6.5 is after return to ground. B. Percentage change from ground pulse rate while in flight treatment for smokers and nonsmokers. Pulse rate was measured along with other physiological measures at each timepoint. C through F are exhaled gas measurements, assayed prior to the beginning of the flight or ground exposure, or at one-hour intervals throughout the flight or ground exposure. Exhaled gases were measured only prior to (hour 0) and during the hypobaric chamber exposure (hours 1-5), not during the final post-exposure timepoint (hour 6.5). C. $ppCO_2$, D. exhaled ppN_2, E. exhaled ppO_2, F. ppH_2O. Bars represent standard error. Asterisks (*) represent nearly significant differences ($p > 0.05$ and < 0.10).

Sample Collection and Processing

Blood and two separate lung cellular samples were taken from each subject in each study phase. Blood samples were collected using PAXgene® blood RNA tubes IVD (Qiagen) and frozen at -80°C. Human Bronchial Epithelial Cells (HBEC) were obtained by bronchoscopy performed under moderate sedation (intravenous midazolam) with bronchial brushing. Three to four separate bronchi were brushed and the cells were rinsed from the brush into 10 ml sterile saline until $5x10^6$ to $1x10^7$ viable cells total were collected as determined by hemocytometer counts for total and viable cells by trypan blue exclusion. HBECs were suspended in Hank's Balanced Salt Solution. Alveolar cells were collected during the same procedure as above by broncho-alveolar lavage (BAL) using 5 x 20 mL aliquots of sterile normal saline in 3 separate bronchopulmonary segments not used for subsequent brushing, discarding returns from the first "bronchial" aliquot (Thompson et al. 1992).

RNA Extraction and Expression Analysis

All cells were collected by centrifugation, resuspended in Qiazol, and placed at -80°C. Ribonucleic acid (RNA) was extracted from blood using PAXgene® Blood miRNA Kit (parts A and B, PreAnalytix, Qiagen) using a QIAcube® (Qiagen) and then stored at -80°C. RNA from cellular samples was purified using RNeasy mini kits (Qiagen) using the miRNeasy mini protocol with a QIAcube® and stored at -80°C. Extracted RNA quality was assessed using RNA 6000 Nano kits (Agilent) on a Bioanalyzer 2100 (Agilent). RNA sample concentrations were determined using a NanoDrop 2000 (Thermo Scientific). Blood and cellular RNA samples were amplified using the GeneChip® WT PLUS Reagent Kit (Thermo Scientific). The concentration of single-stranded cDNA produced by amplification was measured using a NanoDrop 2000 (Thermo Scientific). BAL, HBEC, and blood-derived RNA was amplified into ss-cDNA, fragmented, labeled, and hybridized (ThermoFisher Scientific GeneChip® Hybridization, Wash, and Stain Kit) onto GeneChip Human Transcriptome Assay 2.0 microarrays (ThermoFisher Scientific) for analysis. Microarrays were hybridized for 18 hours, rotating at 60 rpm at 45°C. Chips were then washed and stained using two GeneChip® fluidics station 450 (Affymetrix), using protocol FS450-0001, as per the HTA 2.0 microarray protocol. Stained and washed microarrays were scanned using a 7G-modified GeneChip® Scanner 3000 (Affymetrix).

Data Quality Control and Analysis

Initial quality control was performed for each sample with Affymetrix Expression Console 1.4.1.46. The blood-derived group of .cel file scans consisted of samples from twenty individuals over four timepoints at both flight and ground conditions, for a total of 160 samples. The twenty-individual study group consisted of ten smokers, 5 male and 5 female, and ten nonsmokers, 5 male and 5 female. Seven of the 160 samples collected were found to have low RNA Integrity Numbers (RINs, below 4.5). Electropherograms of these seven samples also indicated poor quality RNA and were excluded from further analyses. The remaining 153 blood-derived samples were used for analysis. Raw .cel files were deposited into the National Center

for Biotechnology Information Gene Expression Omnibus (NCBI GEO) database, accession number GSE120908.

Raw .cel files from blood-derived samples were read into R (version 3.3.1, R Core Team, 2016) together using the R/Bioconductor package oligo (Carvalho and Irizarry 2010) and normalized using "oligo::rma". Quality assessment was performed using the R/arrayQualityMetrics package (Kauffmann et al. 2009) and commands "fitProbeLevelModel" and "arrayQualityMetrics". No significant quality concerns were found and all 153 samples were filtered with the criteria that transcript clusters were retained if their expression in at least one sample exceeded the third quartile value of antigenomic transcript clusters. The R/Bioconductor package limma (Ritchie et al. 2015) was used to determine differential expression between sample subgroups of interest, such as flight vs. ground, smoker vs. nonsmoker, and timepoint comparisons as well as multi-factor comparisons, such as flight smokers vs. ground smokers. Gender and factors not explicitly listed in tables (Tables 1 and 6) were not included in the model due to insufficient power with the small sample size. Also, R/Bioconductor package timecourse (Tai, et al. 2007) was used on blood-derived samples over the four timepoints to rank probesets based on Hotelling T^2 scores.

Samples from HBEC and BAL cells were analyzed separately. These samples were only obtained at timepoint four, and therefore did not include a time series. They were collected at both flight and ground phases. All HBEC samples were found to have RINs greater than 4.5, electropherograms indicated high quality RNA, and all 40 samples were retained. One alveolar sample was found to have a RIN of less than 4.5. The electropherogram also indicated poor quality RNA, and the sample was excluded from analysis. The remaining 39 alveolar samples were retained for analysis. The HBEC and BAL samples were then each separately normalized using the same computational procedure as the blood-derived samples for normalization, quality assessment, and antigenomic filtration. Differential expression detection was performed using R/limma in R for analysis and subgroups of interest.

Differentially expressed (DE) gene sets from normalized and filtered blood, BAL, and HBEC data sets were also analyzed using Ingenuity Pathway Analysis (IPA®, Qiagen). IPA was set to examine DE data sets using the Ingenuity Knowledge Base (genes only) data set as a reference, with interaction and causal networks, "experimentally observed" and "highly predicted" settings for confidence, limited to Human, Mouse, and Rat for species. Node types, data sources, tissues and cell lines, and mutation were set to "all" and data sets used either false discovery rates (HBEC and BAL) or 1/Hotelling T^2 for analysis. Log fold changes of each dataset were used to determine directional Z-scores.

Results and Discussion

Subject Characteristics

Twenty volunteer subjects were selected from among an initial pool of 339 applicants recruited by local advertisement. Two hundred forty-eight subjects were excluded as not meeting the

protocol requirements, including 226 with abnormal pulmonary function test results, 2 ineligible due to a prior lung disease diagnosis, 5 that were outside the age range (25 yrs to 50 yrs), 11 that were infrequent smokers (less than 5 cigarettes daily), and 4 who withdrew from the study. Of the 91 eligible subjects without prior diagnosis, signs, or symptoms of respiratory or cardiac disease (66 non-smokers and 25 smokers), 68 were not age/gender matched or did not complete both phases of the study, and 2 were lost to follow-up.

The final volunteer pool consisted of 10 nonsmokers (complete cigarette/tobacco abstinence) and 10 light smokers (consuming between 5 and 15 cigarettes daily), with 5 males and 5 females in each group. Subject ages ranged from 25 yrs to 47 yrs, with a median of 36 yrs and standard deviation of 7 yrs. Smoking group (S) ages ranged from 25 yrs to 45 yrs, with a median age of 36 yrs and a standard deviation of 7 yrs. The nonsmoking group (NS) ages ranged from 26 yrs to 47 yrs, with a median age of 35 yrs and a standard deviation of 8 yrs. Each of these 20 subjects successfully completed the entire course of the study. Biological samples and physiological data were collected from these subjects for use in further gene expression analysis and in physiological assessments to determine differential effects of altitude exposure on smokers.

Physiological responses among smokers and nonsmokers during flight and ground phases

To examine the physiological status of study participants, SaO_2, pulse rate, and exhaled gas measurements were recorded before and during each hypobaric chamber treatment (Figure 2). Although the differences between smokers and nonsmokers within each condition did not reach statistical significance (Welch 2-sample T-test, 2-tailed, $p < 0.05$), consistent and distinct trends were identifiable between smokers and nonsmokers throughout the course of the flight and ground treatments. SaO_2 during the ground phase remained steady between both groups, varying between 98.0% and 99.1% (Figure 2A). Near-significant differences in oxygen saturation were seen during flight, with mean flight smoker SaO_2 rising above nonsmokers in the last three flight timepoints (Figure 2A, hours 3, 4, and 5 with $p = 0.062$, 0.086, and 0.054, respectively). Smoking and nonsmoking SaO_2 means each returned to 98.7% and 97.7% respectively, following descent. Correspondingly, the average change in pulse rate during the flight phase (100*(flight pulse rate – ground pulse rate/ground pulse rate) increased in the smoking group compared to the nonsmoking group (Figure 2B) and approached statistical significance ($p = 0.078$) at hour 3. Published analyses of SaO_2 and pulse rate in smokers undergoing rapid ascents to altitudes between 12,000 and 15,000 feet show a lack of significant differences in these measures between smokers and nonsmokers upon acute hypoxia, but a significant decrease in SaO_2 over a period of months at altitude (Song et al. 2014, Wu et al. 2012). The sustained increases in pulse rate observed here may signal a physiological response to altitude-induced relative hypoxia in smokers (Peacock 1998, Samuels 2004, Wu et al. 2012).

Exhaled gas measurements revealed differences according to smoking status at both flight and ground conditions, although only one measurement, partial pressure of oxygen (ppO_2) at hour 5) approached statistical significance ($p= 0.099$, Figure 2E), with ground smokers exhaling less

oxygen than ground nonsmokers. Oxygen exhalation varied according to altitude condition in both smokers and nonsmokers, but did not vary significantly between smokers and nonsmokers within an altitude treatment (Figure 2E). Nonsmokers exhaled less CO_2 (Figure 2C) than smokers during both flight and ground phases of the study, in accordance with previous research (Nesthus et al., 1997) although no CO_2 differences were statistically significant. No difference in exhaled nitrogen (Figure 2D) was observed between smokers and nonsmokers during the flight or ground phases. Exhaled water vapor (ppH2O) (Figure 2F) did not demonstrate statistically significant differences between smokers and nonsmokers in either flight or ground conditions, but the smokers exhaled more H_2O than nonsmokers in both phases of the study. Trends toward group differences in gas exhalation existed, particularly in the O_2 and CO_2 measurements, but no significant differences were detected between smokers and nonsmokers within the physiological parameters analyzed for the flight and ground conditions (Figure 2).

Cellular collections reveal differences in total cell numbers

Upon exiting the hypobaric chamber, subjects were transported to a clinical bronchoscopy laboratory (Oklahoma City Veteran's Administration Health Care System Medical Center, Oklahoma City, OK) where final blood samples were collected and bronchoscopies were performed to obtain HBEC and BAL cells as previously described, approximately 1.5 h after altitude or ground exposure. Examination of the cellular content of BAL fluids (Figure 3) revealed significant differences (1-tailed ANOVA with post-hoc Tukey HSD, $p< 0.05$) in total cellular densities between smoking (SG, SF) and nonsmoking (NG, NF) groups at flight and ground within lavage fluids, with smoker BAL fluids containing approximately 2.5 times more cells per mL than nonsmokers (NG= 100,058 cells/mL, NF= 113,336 cells/mL, SG= 315,505 cells/mL, SF= 331,227 cells/mL, NGvSG p= 0.012, NGvSF p= 0.006, NFvSG p= 0.020, NFvSF p= 0.010, Figures 3A and 3B).

BAL fluid was composed of a number of cell types. The majority of BAL cells were mononucleate alveolar macrophages (HAM mono), followed by binucleate alveolar macrophages (HAM bi+), lymphocytes, monocytes, polymorphonuclear cells (PMNs), and ciliated bronchial epithelium (Figure 3C). Mononucleate HAM cells constituted a larger proportion of HAMs than did binucleate HAM cells in all groups, although both mono- and binucleate HAMs differed significantly ($p< 0.05$) between smoking and nonsmoking groups at flight and ground (HAM Mono: NG= 84,071 cells/mL, NF= 98,404 cells/mL, SG= 244,463 cells/mL, SF= 273,064 cells/mL, NGvSG p= 0.018, NGvSF p= 0.004, NFvSG p= 0.036, NFvSF p= 0.009. HAM Bi: NG= 4,641 cells/mL, NF= 4,691 cells/mL, SG= 24,485 cells/mL, SF= 22,044 cells/mL, NGvSG p= 0.003, NGvSF p= 0.012, NFvSG p= 0.004, NFvSF p= 0.013, Figure 3C). Mononucleate and total (mono + bi HAM) HAM densities also demonstrated significant differences between flight and ground smokers and nonsmokers (NG= 88,712 cells/mL, NF= 103,096 cells/mL, SG= 268,949 cells/mL, SF= 295,108 cells/mL, NGvSG p= 0.012, NGvSF p= 0.003, NFvSG p= 0.025, NFvSF p= 0.007, Figure 3C).

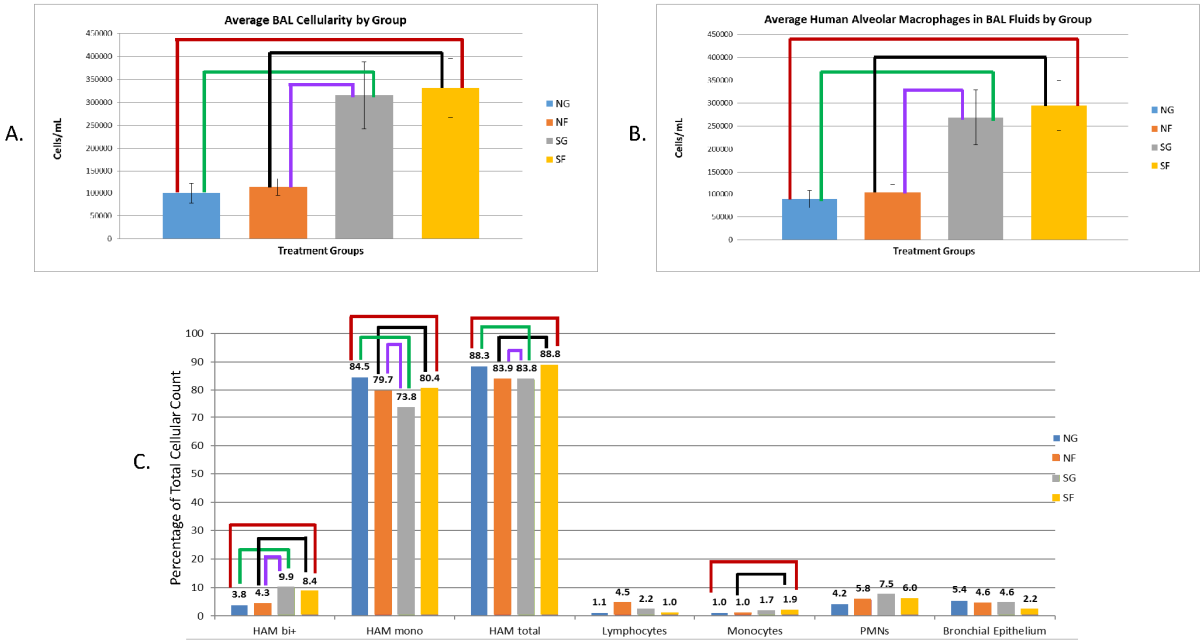


Figure 3. Cell counts and concentrations in cellular lavage samples (BAL cells) and cellular composition of those lavage samples. A. Average concentration of BAL cells (cells/mL) collected from Broncho-alveolar lavages in nonsmokers and smokers from both flight and ground study phases. Columns linked by lines are significantly different ($p<0.05$), individual contrasts are indicated by line color. B. Average concentration (cells/mL) of Human Alveolar Macrophages (HAMs) in BAL samples. Columns linked by lines are significantly different ($p<0.05$), individual contrasts are indicated by line color. C. Average percentage of cellular subpopulations within BAL fluids of each indicated group. Columns linked by lines are significantly different ($p<0.05$), individual contrasts are indicated by line color. All significant differences evaluated by 1-tailed ANOVA with Tukey's post-hoc HSD ($p<0.05$)

Lymphocyte densities showed no significant differences, but increased from 1.1% to 4.5% of cells in nonsmokers during the altitude treatment and decreased from 2.2% to 1.0% of all cells in smokers following the altitude treatment (NG= 1,106 cells/mL, NF= 1,976 cells/mL, SG= 5,919 cells/mL, SF= 4,836 cells/mL, $p>0.05$ in all in comparisons, Figure 3C). Monocyte densities showed significant differences between nonsmokers at ground and flight vs. flight smokers, but not vs. ground smokers (NG= 370 cells/mL, NF= 659 cells/mL, SG= 3,543 cells/mL, SF= 5,070 cells/mL, NGvSF $p= 0.023$, NFvSF $p= 0.037$, Figure 3C). No significant differences were observed in any contrast between smokers and nonsmokers within PMN cells (NG= 3,944 cells/mL, NF= 4,159 cells/mL, SG= 31,570 cells/mL, SF= 17,297 cells/mL, $p>0.05$ in all comparisons, Figure 3C), or ciliated bronchial epithelial cells (NG= 5,220 cells/mL, NF= 3,142 cells/mL, SG= 5,103 cells/mL, SF= 8,846 cells/mL, $p> 0.05$ in all comparisons, Figure 3C). In summary, the total cellular content and composition of BAL fluids in smokers was significantly different in smokers compared to nonsmokers at both altitude conditions, with few exceptions. Average total cellular content of smoker BAL fluids was consistently greater than that of nonsmokers at either altitude condition. The increase in cell recovery from smoker lavages is commonly observed and not due to experimental conditions (Karimi et al. 2012, Heron et al. 2012).

Differential Gene Expression measured by Microarray in BAL and HBEC cells

Total RNA was extracted from BAL and HBECs collected after flight and ground hypobaric chamber treatments. After analyzing initial sample quality (RIN), 39 BAL samples (1 sample was discarded for low quality) and 40 HBEC samples were retained. Total RNA from the selected samples was used to prepare hybridization libraries for analysis by Affymetrix HTA 2.0 microarrays. Following microarray quality control, all transcript clusters (TCs) with at least one sample expressing above the third quartile value of antigenomic microarray probes were selected for differential expression analysis (Blood= 15,086, HBEC= 16,745, BAL= 15,277). Differential expression analysis proceeded using R/limma with duplicate correlation to account for intra-subject variability. Acceptance criteria for differential expression was established as Benjamini-Hochberg adjusted (false discovery rate-FDR) p value of < 0.05.

Initial comparison of RMA-summarized and antigenomic-filtered results revealed large-scale heterogeneity within the subject population for both BAL and HBECs. Principal components analysis (PCA) of the summarized datasets demonstrated no clear separation between any known group, revealing a large amount of variation based upon all factors measured (Figure 4A and D). However, unsupervised hierarchical clustering by heatmap with the same datasets demonstrated a more apparent, but imperfect, separation of individual samples into smoking and nonsmoking groups (Figure 4B and C). In the HBEC cells, only one non-smoker sample clustered with the smoking samples, and two outlying smoker samples did not cluster with either group (Figure 4B and C). The BAL cells did not cluster as strictly as the HBEC, although smokers largely grouped together, as did a subset of the nonsmoker population. This looser clustering potentially may reflect the more heterogeneous cellular composition of the lavage fluid in comparison to the HBEC population recovered by small airway brushing. Samples from individual subjects clustered together on the same terminal branch in BAL samples in 16 of 20 instances, surpassing the individual-based homogeneity (13 of 20 subjects clustering together) of the HBEC samples. Thus, the majority of variance between samples was based upon inter-subject variance, followed by variance between smokers and nonsmokers. Variation between samples based on flight and ground status was not detectable in either PCA or clustering analyses.

Comparisons of flight versus smoking status detected significant transcriptional changes in both BAL and HBEC cells (Table 1). No significant differential gene expression between flight and ground conditions (FvG) was observed. Comparison between smokers and nonsmokers (SvN) yielded 836 upregulated and 802 downregulated genes in BAL cells compared to 3,424 up- and 3,548 downregulated genes in HBEC cells. Comparison of smokers and nonsmokers during simulated flight (FSvFN) yielded 405 up- and 306 downregulated genes in BAL and 2,142 up- and 2,650 downregulated genes in HBEC cells, whereas comparison of ground smokers and nonsmokers (GSvGN) detected 286 up- and 399 downregulated genes in BAL and 1,548 up- and 1,696 downregulated genes in HBECs.

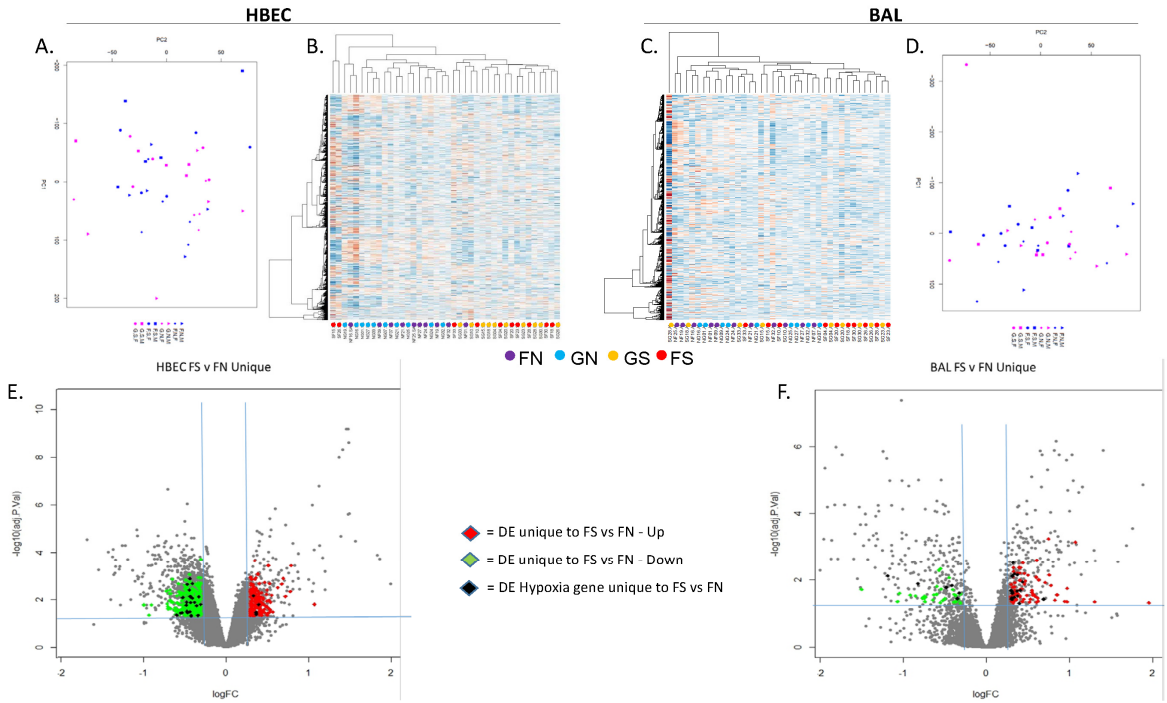


Figure 4. Gene expression in cellular populations as a result of condition and treatment. Gene expression as measured by microarray analysis was compared globally by Principal Component Analysis and unsupervised hierarchical clustering, and differential expression was assessed between smokers and nonsmokers in flight, and distinguished from these genes differentially expressed between smokers and nonsmokers at ground elevation. A. Principal component analysis (PCA) of all HBEC samples utilized. B. Heatmap-based hierarchical clustering of HBEC samples. Rows are transcript clusters, columns are samples. C. Heatmap-based hierarchical clustering of BAL samples. Rows are transcript clusters, columns are samples D. PCA of BAL samples. E. Volcano plot describing the –log10 (adj.Pval) vs. log fold change of HBEC samples comparing flight smokers vs. flight nonsmokers after purging to remove DE genes in ground smokers vs. flight nonsmokers. F. Volcano plot examining differential expression in BAL cell samples, also contrasting –log10 (adj.Pval) vs. log fold change of BAL samples after purging to remove DE genes in ground smokers vs. ground nonsmokers. Transcript clusters highlighted in red and green are not present in the GS vs. GN comparison, and are upregulated and downregulated, respectively. Transcript clusters highlighted in black are hypoxia-related genes unique to the FS vs. FN comparison. Differential expression criteria are adjusted P value (FDR) <0.05 and log fold change >|0.3|. F=Flight, G=Ground, S=Smoker, N=Nonsmoker, F (following second comma)=Female, M (following second comma)=Male.

To determine the specific effects of flight on smokers, the FSvFN differentially expressed (DE) TC list was purged of those TCs appearing in the GSvGN list, resulting in a set of genes transcriptionally altered in smokers only during flight. This list was further restricted by adding an additional acceptance criterion for differential expression of a log2 fold change exceeding > |0.3|. According to these criteria, 187 genes were up- and 361 genes down-regulated in HBEC cells, (Figure 4E, Table 1, Supplementary Table 1), while 89 genes were up- and 47 down-regulated in BAL cells specifically in smokers during flight (Figure 4F, Table 1, Supplementary Table 2). Unsupervised hierarchical clustering of each DE gene list by cell type revealed imperfect sample clustering, but placed the majority of smokers within the same hierarchical branch in each cell type (Figure 5 A and B). These genes were significant only during flight, not

during the ground phase, and so represent genes that are differentially expressed in smokers solely due to exposure to a hypobaric environment during the flight phase of this study.

Differential Expression in BAL and HBEC Cells				
	BAL		HBEC	
Contrast	Upregulated	Downregulated	Upregulated	Downregulated
F - G	0	0	0	0
S - NS	836	802	3,424	3,548
FS - GS	0	0	0	0
FN - GN	0	0	0	0
FS - FN	405	306	2,142	2,650
GS - GN	286	399	1,548	1,696
FS-FN Unique (Purged List)	89	47	187	361

Table 1. Differential Expression count resulting from comparisons between altitude treatment and smoking status. Differential expression is defined as genes differing between the indicated comparisons with a Benjamini-Hochberg Adjusted p-value (FDR) < 0.05 and a log fold change > |0.3| in either direction. F=Flight, G=Ground, S=Smoker, N=Nonsmoker.

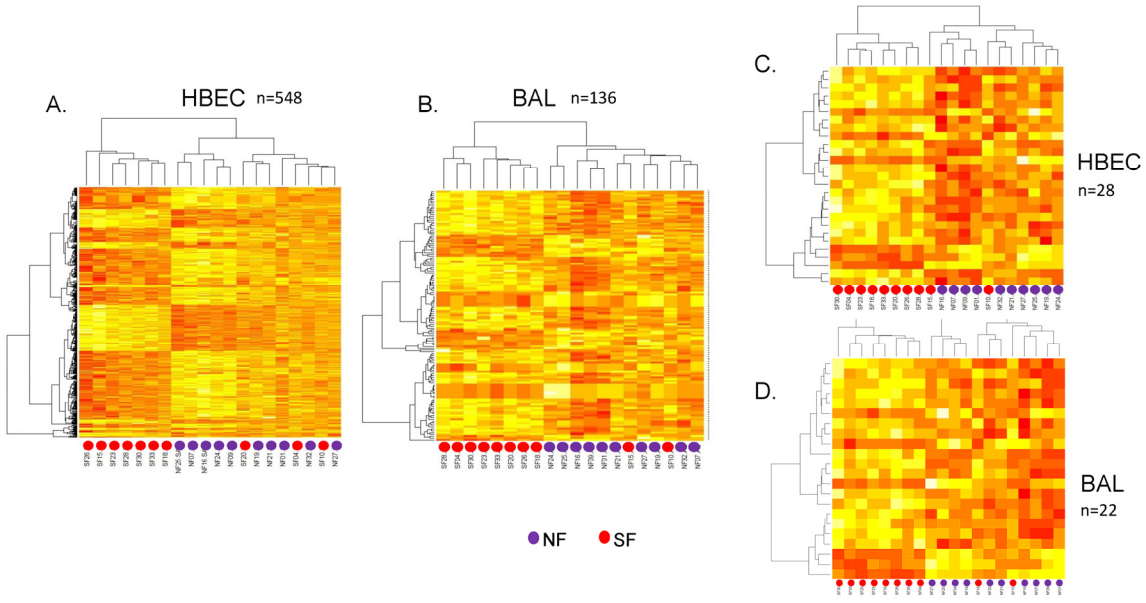


Figure 5. Differentially expressed and hypoxia-limited gene lists show distinct gene expression patterns based on smoking status in HBEC and BAL cells. Flight samples from smokers and nonsmokers were clustered according to expression patterns in differentially expressed and hypoxia-limited differentially expressed gene sets. A. Unsupervised hierarchical clustering-based heatmap utilizing Differentially Expressed Genes in HBEC cells (n=548, FDR<0.05, LFC > |0.3|) between Smokers and Nonsmokers in Flight. B. Differentially Expressed Genes in BAL cells between Smokers and Nonsmokers (n=136, FDR<0.05, LFC>|0.3|) in Flight. C. Differentially expressed Hypoxia-involved genes in the HBEC gene list ((n=27, FDR<0.05, LFC>|0.3|). D. Differentially-expressed hypoxia-related genes in the BAL gene list ((n=28, FDR<0.05, LFC>|0.3|).

Cell Types Display Differing Expression Patterns in Smokers During Flight

Comparing the differentially expressed (DE) transcript cluster (TC) lists (defined as the DE transcript clusters of the FS vs. FN contrast purged of DE GS vs. GN transcript clusters) among both cellular types and blood (discussed in following sections), we found that no DE genes were common to all analyses, or were common to blood and BAL cells (Figure 6). Six DE transcript clusters were common to HBEC cells and blood, representing 5 regulatory RNAs; *SCARNA6* (2 TCs), the long non-coding RNAs (lncRNA) *TCONS_00025791* and *uc004coz.1*, *U4atac small nuclear RNA 12*, and *SNORD116-2*. Only one gene, NFκB inhibitor alpha (*NFKBIA*, corresponding to transcript cluster TC14001036.hg.1), was common to HBEC and BAL data sets, and was downregulated at similar magnitudes in each (-0.41 LFC in HBEC cells, -0.32 LFC in BAL cells). The NFKBIA protein inhibits NFκB (Jacobs and Harrison 1998) and is degraded by phosphorylation during hypoxia, thereby allowing NFκB to bind its DNA targets (Koong et al. 1994), but can also be upregulated during hypoxia (Fang et al. 2009, Shin et al. 2006). Thus, downregulation of *NFKBIA* in the current study may in fact signal a reduced hypoxic response in smokers during flight. The global disparity of DE transcript clusters between sample types indicates the generation of differing responses to the same mildly hypoxic stimulus.

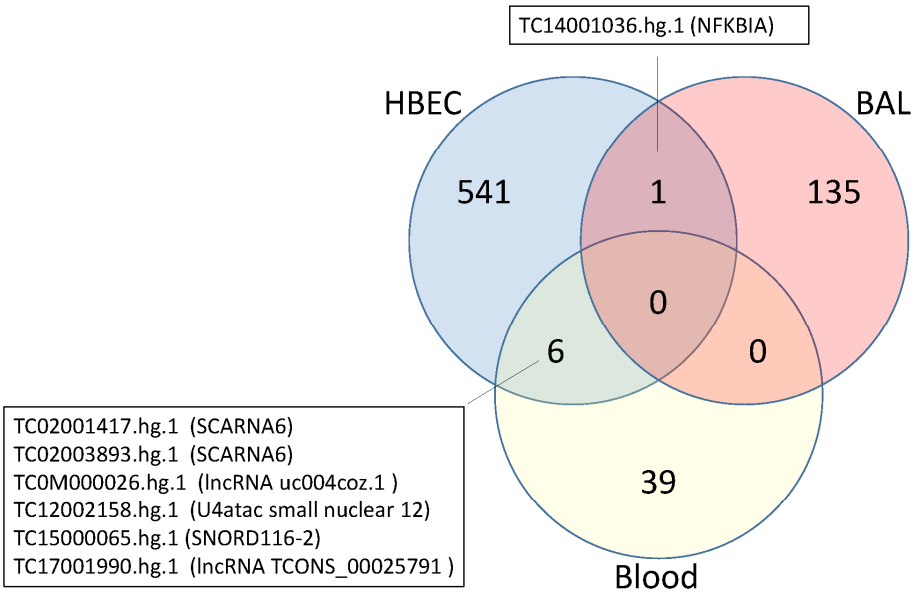


Figure 6. Venn diagram detailing differential expression similarities between HBEC, BAL, and blood samples. Intersecting genes are indicated in text boxes linked to intersect by lines.

To examine the involvement of hypoxia-related genes in the differential response of smokers to mild flight-induced hypoxia, we isolated subsets of hypoxia-related genes (based on hypoxia-termed human genes downloaded from NCBI on 2018_02_18) from the HBEC and BAL DE lists

(Table 2) and performed unsupervised hierarchical clustering and heatmap analysis of each (Figure 5 C and D). The two gene lists, with one DE gene in common (*NFKBIA*), each display differing expression patterns. DE hypoxia genes are mainly upregulated in BAL cells (16 of 22 DE hypoxia genes upregulated, Table 2), but mostly downregulated in HBEC cells (24 of 28 DE hypoxia genes downregulated, Table 2).

Flight-Responsive Differentially Expressed Hypoxia-Related Genes in Smokers vs. Nonsmokers During Flight

	Transcript Cluster ID	logFC (- | +)	adj.P.Val	Gene Symbol
BAL	TC16000148.hg.1		0.00295	ABAT
	TC12001227.hg.1		0.006488	YBX3
	TC01005362.hg.1		0.006918	PTAFR
	TC17000408.hg.1		0.007522	CCL4
	TC20000245.hg.1		0.007642	ACSS2
	TC22001480.hg.1		0.011247	ADA2
	TC02002445.hg.1		0.013007	NR4A2
	TC06004141.hg.1		0.014346	SOD2
	TC10001182.hg.1		0.016506	NAMPTP1
	TC15001484.hg.1		0.020252	ADAM10
	TC19001593.hg.1		0.020737	PLAUR
	TC10001166.hg.1		0.021394	NRP1
	TC21000538.hg.1		0.02261	ITGB2
	TC14001036.hg.1		0.02441	NFKBIA
	TC01002401.hg.1		0.024832	SLC9A1
	TC19002442.hg.1		0.030103	CEBPA
	TC21000249.hg.1		0.032958	COL6A1
	TC11000589.hg.1		0.034615	VEGFB
	TC11002074.hg.1		0.035871	UCP2
	TC16000374.hg.1		0.037344	ITGAM
	TC01005354.hg.1		0.038234	SLC9A1
	TC06002126.hg.1		0.049698	SGK1

	Transcript Cluster ID	logFC (- | +)	adj.P.Val	Gene Symbol
HBEC	TC03002268.hg.1		0.001386	TGFBR2
	TC08001683.hg.1		0.002235	AGO2
	TC01001090.hg.1		0.002471	TXNIP
	TC16001794.hg.1		0.004094	SMG1
	TC01006037.hg.1		0.00737	TNNT2
	TC16001943.hg.1		0.007761	SMG1P7
	TC03003047.hg.1		0.008003	GSK3B
	TC16001001.hg.1		0.008576	SMG1P6
	TC16000944.hg.1		0.008576	SMG1P4
	TC14001036.hg.1		0.009316	NFKBIA
	TC01005816.hg.1		0.013242	MCL1
	TC09001793.hg.1		0.013758	SMARCA2
	TC06001027.hg.1		0.013832	TNFAIP3
	TC18000213.hg.1		0.016895	PMAIP1
	TC0X001064.hg.1		0.016921	SMC1A
	TC06003084.hg.1		0.018062	TNFAIP3
	TC09002591.hg.1		0.019393	HSPB1P1
	TC14000471.hg.1		0.032615	FOS
	TC11002857.hg.1		0.034525	ATM
	TC07001318.hg.1		0.036093	INHBA
	TC0M000026.hg.1		0.036672	none
	TC17000728.hg.1		0.036887	MIR21
	TC05002424.hg.1		0.036941	SMN2
	TC04002840.hg.1		0.039448	HPGD
	TC0M000011.hg.1		0.043872	none
	TC05000701.hg.1		0.046505	EGR1
	TC03002006.hg.1		0.048352	TNFSF10
	TC06003070.hg.1		0.049853	MYB

Table 2. Bars indicate magnitude and direction of differential regulation (log fold-change) of indicated genes in each cell type. Red bars indicate upregulation in FS versus FN, green bars indicate downregulation in FS versus FN.

The genes *CCL4*, *NR4A2*, *SOD2*, *NAMPTP1*, *NFKBIA*, and *UCP2* were downregulated in the BAL FS vs. FN comparison. *CCL4*, *NRA4A2*, and *SOD2* are known to be upregulated during hypoxia, and all play roles in promoting the cellular hypoxic response (Wang et al. 2016, Leonard et al. 2008, Kenney et al. 2012, Liu et al. 2006). *NAMPTP1*, a pseudogene, has no clear role in smoking or hypoxia, but *NAMPT* is ontologically linked to hypoxia. *NFKBIA*, as previously discussed, increases during hypoxia but its protein is preferentially degraded, thereby allowing increased NFκB activity (Shin 2009). *NFKBIA* downregulation, as previously discussed, likely indicates a downregulation of hypoxic effectors in BAL cells (Koong 1994, Shin et al. 2006, Fang et al. 2009). Of the upregulated genes with clear, published roles in hypoxia, *UCP2*, *YBX3*, and *VEGFB* all play roles in mitigating cellular damage or increasing oxygen availability (Deng et al. 2012, Nie et al. 2012, Morfoisse et al. 2015). *ABAT*, also upregulated (Table 2), is only linked ontologically to

hypoxia, catabolizes the hypoxia-induced inhibitory neurotransmitter *GABA* which increases during hypoxia, and thus may be upregulated in response to a hypoxia-linked increase in *GABA* (Parviz 2016, Nillson and Winberg 1993).

Only two of the upregulated HBEC hypoxia transcript clusters are assigned to known genes (*TNNT2* and *PMAIP1*). The unassigned transcript clusters are each classified by Affymetrix (http://www.affymetrix.com/analysis/index.affx) as lncRNAs. Of the upregulated hypoxia genes in HBEC cells, *TNNT2* (*cTNT*) is a marker of hypoxia-induced cardiac injury that is elevated in the blood during asphyxia (Rajakumar et al. 2008). *PMAIP1* (*NOXA*) transcription is activated by HIF1A, promotes the p53-mediated apoptotic pathway, and functions to mitigate *NFKBIA*-mediated inflammation in airway epithelial cells (Villunger et al. 2003, Kim et al. 2004, Zhang et al. 2018). The increased expression of *PMAIP1* may partially explain the apparent absence of an inflammatory response in smoker BAL cells. The downregulated hypoxia genes in HBEC cells include *FOS*, *ATM*, *miR21*, *EGR1*, and *AGO2*, known hypoxic response effectors that are typically upregulated during hypoxia (Muller et al. 1997, Bencokova et al. 2009, Liu et al. 2014, Chen et al. 2013, Shen 2013). The downregulation of both *AGO2* and *miR21* is interesting, as the AGO2 protein binds *miR21* RNA, and the *mir21*-AGO2 complex functions to cleave *miR21* mRNA targets. Thus, downregulation of both *miR21* and *AGO2*, which binds directly to *miR21* and is required for *miR21* activity, may signal a coordinated downregulation of *miR21*-based responses, thereby decreasing the preferential cleavage of *miR-21* targets (Lima et al. 2009, Mace et al. 2013, Teteloshvili et al. 2017). These data suggest smoker HBEC cells exhibit an inhibition of the hypoxia response during flight.

To determine the ability of the DE hypoxia genes to distinguish between samples, unsupervised hierarchical clustering by heatmap was performed on both BAL and HBEC data sets. As seen previously with DE gene-based subsets, the hypoxia-related DE gene lists do not perfectly distinguish between smokers and nonsmokers, and individual effects are still seen (Figure 5C and D). However, two clear expression patterns were observed that clearly distinguish smokers and nonsmokers during flight in both cell types. In each, one distinct branch of each clustering analysis encompasses the majority of smokers, while the second main branch includes all of the nonsmokers, but includes a minority of smokers. Thus, the effect of smoking in flight is distinct and separate from that of nonsmokers, but a minority of smokers exhibit a diffuse expression pattern grouped on a separate stem of the nonsmoking branch. The basis of these intermediate responses are not clear, but we speculate they are due to the overwhelming effect of the individual within the study.

IPA-detected Canonical Pathways Differ Between BAL and HBEC cells in Smokers During Flight

To examine the functional implications of the differing expression profiles observed between cell types, each purged FS vs. FN DE gene list was submitted to Ingenuity® Pathway Analysis (Qiagen, Hilden, Germany). Only a portion of the DE genes identified in each dataset were suitable for analysis (Blood=15, HBEC=191, BAL=96); the remaining DE genes did not possess sufficient functional information in IPA for analysis. No duplication was observed among the

top 5 canonical pathways seen for either of the cell types (Table 3), likely reflecting the differing cellular composition of each cellular source. Thirty-six and 56 significant canonical pathways were identified in the BAL and HBEC DE gene lists, respectively, with only two in common; IL17a signaling in Airway Cells and Glucocorticoid Receptor signaling. The large-scale difference in detected pathways further demonstrates the divergence of responses to hypoxia between BAL and HBEC cells.

Among the top 5 canonical pathways in the HBEC comparison (Table 3), only EIF2 signaling was assigned a directional Z-score (z = -1.342), the negative score indicating suppression. EIF2 promotes hypoxia tolerance and adaptation by suppressing translational initiation and is activated in response to infection (reviewed by Simon et al. 2008, Shrestha et al. 2012), signaling a suppression of the hypoxia and pathogenesis responses in smokers during the flight treatment. The other top 4 detected pathways, IL17a signaling in fibroblasts, TNFR2 signaling, CD40 signaling, and Human Embryonic Stem Cell signaling, all typified by downregulation of the individual genes comprising each pathway (Supplementary Table 3), indicate a global downregulation of the hypoxia response in HBEC cells.

Table 3. Top 5 Differentially Regulated IPA® Canonical Pathways in Smokers vs. Nonsmokers During Flight

	Canonical Pathway	p-Value
Bronchio-Alveolar Lavage Cells	Complement System	0.0000132
	Granulocyte Activation and Diapedesis	0.000733
	Leukocyte Extravasation Signaling	0.00145
	Caveolar-Mediated Endocytosis Signaling	0.00274
	Acute Phase Response Signaling	0.00451
Human Bronchial Epithelial Cells	EIF2 Signaling	0.0000528
	IL-17A signaling	0.0000534
	TNFR2 Signaling	0.000734
	CD40 Signaling	0.00125
	Human Embryonic Stem Cell Signaling	0.00163
Blood	Interferon Signaling	0.00933
	ATM signaling	0.0257
	Gαs Signaling	0.0286

Among the top 5 BAL canonical pathways (Table 3), only the Leukocyte Extravasation signaling pathway, an inflammation-responsive pathway resulting in immune cell recruitment to the affected site (reviewed by Langer and Chavakis 2009), possessed a Z-score (Z = +2.0) signaling an increase in leukocyte extravasation. When considered in combination with the significantly enriched, but directionless (Z-score not determined), Complement System, Granulocyte Adhesion/Diapedesis, Caveolar-Mediated Endocytosis signaling, and Acute Phase Response Signaling pathways, the BAL cell response to flight in smokers appears to activate immune induction and leukocyte recruitment to the alveoli (Supplementary Table 3).

Upstream Regulatory Effectors Differ Between BAL and HBEC cells in Smokers During Flight

IPA analysis identified 10 significantly altered upstream regulatory molecules in HBEC cells (4 activated, 6 suppressed) and 4 in BAL cells (1 activated, 3 suppressed). *NFκB1* and *TLR4* were each predicted to be suppressed in both cell types (Table 4, Figure 7), suggesting that both cell types are responding to hypoxia, though not in identical fashion, as not all the same genes were modulated by altitude. In BAL cells, suppression of *NFκB* and *TLR4* was predicted by the downregulation of *CCL4*, *C3, SOD2*, and *NFKBIA*, and by upregulation of *GPR34*.

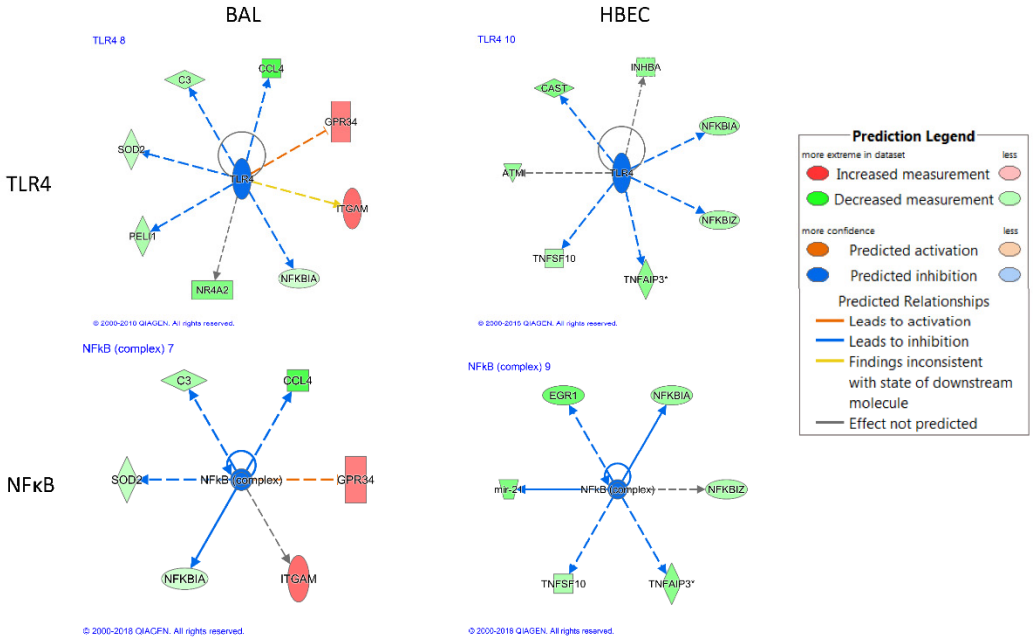


Figure 7. Upstream regulator networks in common between BAL and HBEC cells in smokers during flight in IPA®. Significant DE genes from BAL and HBEC cells were presented to IPA and the effect of these genes on upstream regulatory elements was predicted based on reported log fold-change of each measured gene. *TLR4* and *NFκB* are predicted to be downregulated in both cell types. (*) = *TNFAIP3* represented by two transcript clusters. Intensity of red/green coloration represents magnitude of up- or down-regulation, respectively.

Downregulation of *TLR4* additionally was predicted by suppression of *NR4A2* and *REL1* (Figure 7). In HBEC cells, *TLR4* and *NFκB* were predicted to be suppressed by the observed downregulation of *NFKBIA*, *NFKBIZ, TNFAIP3*, and *TNFSF10*, with *TLR4* suppression also predicted through downregulation of *ATM*, *CAST*, and *INHBA*, while suppression of *NFκB* was additionally predicted through downregulation of *mir-21* and *EGR1* (Figure 7). As *TLR4* expression is expected to increase during hypoxia (Kim et al. 2009, Wu G et al. 2018), its relative suppression in smokers during flight indicates an inhibited hypoxic response in both cell types. The predicted suppression of *NFκB*, a transcriptional activator which acts on HIF1A and other hypoxia-responsive factors (Koong et al. 1994, reviewed by D'Ignazio et al. 2016), in both cell types further suggests downregulation of the hypoxic response in smokers during flight.

Table 4. IPA® Predicted Upstream Regulators Among DE Genes in HBEC and BAL Flight Smokers

	Upstream Regulator	Molecule Type	Pred. Act. State	Act. z-score	p-value overlap	Target molecules
BAL	**NFkB (complex)**	complex	Inhibited	-2.223	4.61E-03	C3,CCL4,GPR34,ITGAM*,NFKBIA, SOD2
	TLR4	Transmem. receptor	Inhibited	-2.202	4.69E-05	C3,CCL4,GPR34,ITGAM,NFKBIA, NR4A2*,PELI1,SOD2
	AHR	ligand-dep. nuclear receptor	Inhibited	-2.219	7.33E-03	CEBPA,COL6A1,PI4K2A,SOD2,VCL
	KMT2D	Transcr. regulator	Act.	2	5.81E-03	GALNT12,GUSB,MARVELD1,SMAD6
HBEC	**NFkB (complex)**	complex	Inhibited	-2.207	2.99E-02	EGR1,mir-21, NFKBIA,NFKBIZ*,TNFAIP3,TNFSF10
	TLR4	Transmem. receptor	Inhibited	-2.2	3.53E-03	ATM*,CAST,INHBA*,NFKBIA,NFKBIZ, TNFAIP3,TNFSF10
	MYD88	other	Inhibited	-2.402	2.90E-03	ATM,EGR1,INHBA,NFKBIA,NFKBIZ, TNFAIP3
	TICAM1	other	Inhibited	-2.2	1.30E-03	EGR1,NFKBIA,NFKBIZ,TNFAIP3, TNFSF10
	TGFB1	growth factor	Inhibited	-2.668	1.64E-02	FOS,GLDN,HCAR2,INHBA,mir-21, mir-320,mir-500,NFKBIA*,TGFBR2
	IFNG	cytokine	Inhibited	-2.261	2.76E-03	B2M,GLDN,HCAR2,NFKBIA*,NFKBIZ, PMAIP1,POMC,PRPF8,SCNN1B, TGFBR2,TNFSF10,TXNIP
	Hdac	group	Act.	2.057	2.23E-03	EGR1,FOS,TGFBR2,TNFSF10,TXNIP
	RICTOR	other	Act.	2.236	2.46E-02	EGR1,RPL13A,RPS13,RPS3,RPS5
	CEBPB	Transcr. regulator	Act.	2.219	2.36E-03	ATM,FHL1,FOS*,GSK3B,HBB*,POMC, SLC38A2
	TP73	Transcr. regulator	Act.	2.19	4.05E-03	B2M*,EGR1*,LYZ,PMAIP1,RPRM, SERPING1,STMN1

* Direction of upstream regulator effect on indicated gene not determined. Underlined genes indicate direction of expression not consistent with known regulator effect on that gene, i.e. predicted direction is opposite of measured direction. Red text indicates measured upregulation, blue text indicates measured downregulation. Yellow background = activated upstream regulator. Green background = inhibited upstream regulator.

The remaining altered upstream regulators in HBEC cells further indicated a suppression of the hypoxic response. Suppression of *MYD88*, which promotes a TLR4-mediated inflammatory response to hypoxia that stabilizes HIF1a (Jantsch et al. 2011), indicates suppression of hypoxia-related inflammation. Suppression of *TICAM1* (*TRIF*), involved in the Toll-like receptor 3 (*TLR3*) response to hypoxia and promoting cellular survival during hypoxia (Pimentel-Coelho et al. 2013); *TGFB1*, which promotes cellular survival under hypoxic conditions (Suzuki et al. 2006); and *IFNG*, which exhibits decreased expression during hypoxia in T-cells in a HIF1a-independent manner (Roman et al. 2010); present a combined set of evidence favoring downregulation of the hypoxic response in HBEC cells. Conversely, the upregulation of *RICTOR*, which promotes *HIF1A* expression and *VEGF* secretion (Schmidt et al. 2017); *HDAC*, involved in upregulating *HIF1A* expression (Kong et al. 2006); *CEBPB*, also inducing *HIF1A* expression (Yamaguchi 2015); and *TP73*, upregulated during hypoxia and promoting or suppressing vascularization depending on its splicing state (Marin and Marques 2016, Sabapathy 2016), indicate a drive by HBEC cells to increase *HIF1A* expression and prime the hypoxic response. This mixed down- and up-regulation of hypoxia-inducible upstream regulators in HBEC cells may indicate a downregulation of the hypoxia response along with an increase in regulatory molecules (*HDAC*, *RICTOR*, *CEBPB*, and *TP73*) in a cellular attempt to mitigate the damage resulting from a suppressed hypoxic response and to "jump-start" a *HIF1A*-mediated cascade.

Conversely, BAL cells in smokers during flight demonstrate upregulation of the cellular damage responses in contrast to nonsmokers. Of the remaining altered upstream regulators in BAL cells, *AHR*, which regulates a xenobiotic-response pathway sharing some elements in parallel with the HIF1a pathway (Zhang and Walker 2007), may be downregulated as an accessory to the relative downregulation of the hypoxic response seen in BAL cells. *KMT2D*, activated in BAL cells, is a histone H3K4 methyltransferase that dimethylates the promoters and enhancers of hypoxia-reoxygenation/cell cycle-related genes, leading to increased expression of hypoxia-related genes (Ang et al. 2016), perhaps representing a response in smoker BAL cells to increase hypoxia-related gene expression.

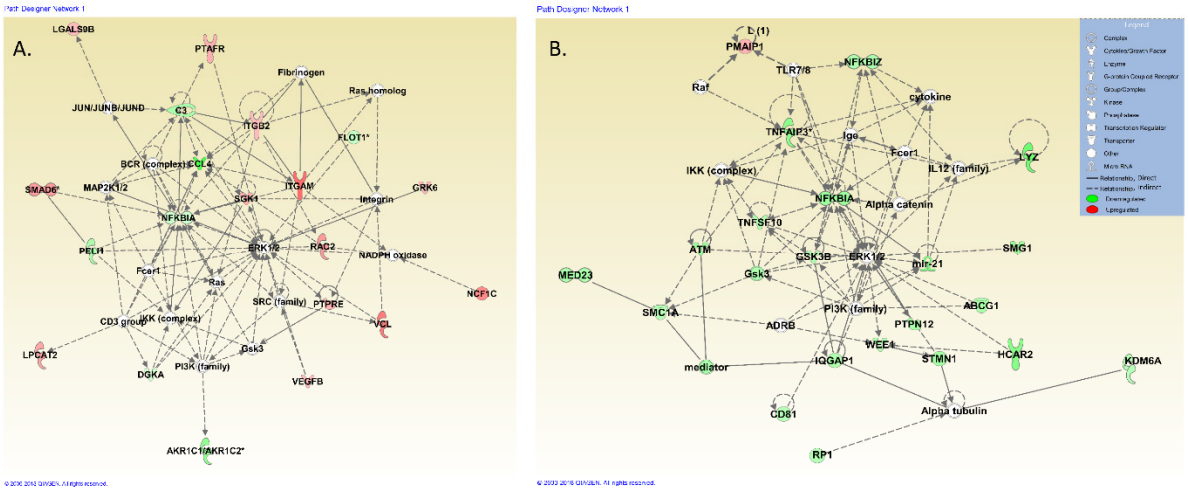
Identified Molecular Networks Indicate that Differing Cellular Expression Patterns Stem from Similar Stimuli

Molecular network identification in IPA, utilizing the previously-described purged FS vs. FN gene lists, resulted in the identification and assembly of 17 networks for HBEC cells and 7 networks in BAL cells (Table 5). The diseases and functions predicted for HBEC cells are largely represented by cell death, cancer, and lipid metabolism-related networks, while those identified in BAL cells relate to inflammatory responses, cancer, and embryonic/cellular development (Table 5). Examination of the top-scoring network identified in each cell type revealed a number of similarities in regulatory targets, although with a large difference in individual components (Figure 8). Both top networks in BAL and HBEC cells described networks converging on *ERK 1/2* (not DE in either) and *NFKBIA* (downregulated in both).

Table 5. Identified IPA® Networks Among HBEC and BAL cells.

	Network ID	Score	DE Molecules	Top Diseases and Functions
HBEC	1	37	21	Cell Death and Survival, Cellular Development, Cellular Growth and Proliferation
	2	28	17	Cancer, Cell Death and Survival, Organismal Injury and Abnormalities
	3	21	14	Gene Expression, Cell Death and Survival, Neurological Disease
	4	17	12	Cancer, Gastrointestinal Disease, Organismal Injury and Abnormalities
	5	17	12	Cancer, Organismal Injury and Abnormalities, Reproductive System Disease
	6	17	12	Endocrine System Development and Function, Organismal Functions, Endocrine System Disorders
	7	14	10	Lipid Metabolism, Molecular Transport, Small Molecule Biochemistry
	8	12	9	Lipid Metabolism, Molecular Transport, Small Molecule Biochemistry
	9	2	1	Cell Morphology, Cellular Assembly and Organization, Developmental Disorder
	10	2	1	Cellular Development, Cellular Growth and Proliferation, Organ Development
	11	2	1	Developmental Disorder, Hereditary Disorder, Neurological Disease
	12	2	1	Cardiovascular Disease, Organismal Injury and Abnormalities, Reproductive System Disease
	13	2	1	Cell Cycle, Cell Morphology, Cancer
	14	2	1	RNA Post-Transcriptional Modification, Cancer, Gastrointestinal Disease
	15	2	1	Carbohydrate Metabolism, Lipid Metabolism, Small Molecule Biochemistry
	16	2	1	Connective Tissue Disorders, Developmental Disorder, Hereditary Disorder
	17	1	1	Behavior, Endocrine System Disorders, Hereditary Disorder
BAL	1	38	20	Cellular Compromise, Inflammatory Response, Cellular Movement
	2	31	17	Molecular Transport, Cardiovascular Disease, Organismal Injury and Abnormalities
	3	22	13	Cancer, Neurological Disease, Organismal Injury and Abnormalities
	4	19	12	Embryonic Development, Nervous System Development and Function, Organ Development
	5	15	10	Cellular Development, Cancer, Organismal Injury and Abnormalities
	6	13	9	DNA Replication, Recombination, and Repair, Inflammatory Response, Organismal Injury and Abnormalities

	7	2	1	Cardiovascular System Development and Function, Cell Cycle, Cellular Development
Blood	1	8	3	Cell Morphology, Cellular Function and Maintenance, DNA Replication, Recombination, and Repair



BAL HBEC

Figure 8. Top-scoring networks in HBEC and BAL cells display differing transcriptional responses regulating *ERK1/2*. A. HBEC cells display an inhibited response in most identified genes regulating *ERK1/2*. BAL cells exhibit upregulation of the majority of genes regulating *ERK1/2*. Molecules highlighted in green are downregulated, white molecules have no direction-of-change information in the data set supplied to IPA

The individual molecules acting on these networks were largely different, however. HBEC cells showed downregulation of most DE molecules (21 molecules downregulated) but the upregulation of only one, *PMAIP1*. In contrast, BAL cells showed large-scale activation of immune response-related molecules (12 molecules upregulated) and downregulation of 6 molecules, including *FLOT1*. In murine embryonic stem cells, *FLOT1* was upregulated in wild-type cells during hypoxia (5% O_2), but suppressed under the same hypoxic condition in *HIF1a*-null, but not *HIF2a*-null, cells (Hu et al 2006). Thus, *FLOT1* downregulation corresponds to the predicted downregulation of *HIF1a* activity. Considered in conjunction with the large-scale downregulation of hypoxia-related genes (Figure 3, Table 6), HBEC cells in flight smokers appear to exhibit an inhibited response to aviation-related hypoxia that results in the activation of cell death and alternate metabolic responses. BAL cells, in response to the same hypoxic stimulus, display an increase in inflammatory, organismal injury, molecular transport, and cellular development responses (Table 5).

Table 6. Differential Expression Among Blood Sample Contrasts				
Contrast	**R/timecourse**		**R/limma (FDR<0.05)**	
	Number Changed	**T^2 Criteria**	**Up**	**Down**
F-G	1	>20	0	0
FS - FN	48	>20	0	0
GS - GN	84	>20	0	0
(FS-FN)-(GS-GN)	45	>20	0	0
FS - FN (Female)	399	>20	0	2
GS - GN (Female)	198	>20	0	1
FS - FN (Male)	240	>20	2	6
GS - GN (Male)	476	>20	0	0

In order to better appreciate the global changes occurring within BAL and HBEC cells, we collapsed all the accumulated data into heatmap-based representations of affected Diseases and Functions, with individual cells representing the magnitude of suppression or activation based on the measured expression of individual genes in each dataset (Figure 9). Examining the functions in common between the two cell types illustrate differing expression profiles. The "Organismal Injury and Abnormalities" (red box, Figure 9) function was similar in both cell types, but exhibiting a larger number of individual processes in HBEC cells. The "Cellular Development", "Hematological System Development", "Cellular Growth and Proliferation", and "Hematopoiesis" (black, light green, yellow, and dark green boxes respectively, Figure 9) functions were largely upregulated in BAL cells and overwhelmingly downregulated in HBEC cells. Within the diverging functions, HBEC cells showed upregulation of cell death and cancer related functions and decreases in cellular growth functions, while BAL cells showed increases in cellular signaling, growth, and injury responses.

Differentially Expressed Genes in Blood Samples

Blood samples collected for each individual in both flight conditions at the timepoints described (Figure 1) were analyzed for changes in gene expression over time. Initial clustering analysis of the data using PCA produced no discernable clustering (not shown). Due to the large number of potential contrasts in this phase of the study (including 4 timepoints, two conditions, two separate altitude levels within the flight phase – ground level at timepoints 1 and 4, and 8,000 ft. at timepoints 2 and 3, gender, and smoking status), heatmap-based hierarchical clustering was not performed. As this study was intended to produce broadly applicable biomarker genes for use in a wide population, all data sets were summarized together and analyzed by both R/limma (using duplicate correlation to account for intra-subject variation) and R/timecourse analysis (using the paired sample method to account for intra-sample variance, and imputing values for missing timepoints). Since R/limma contrasts found few differentially expressed

genes in the contrasts of most interest (FvG and SvN), and this study's aim was initial biomarker discovery where false negatives present a greater concern than false positives (false positives being able to be discarded in future biomarker validation efforts), analysis focused on results from R/timecourse (Table 6).

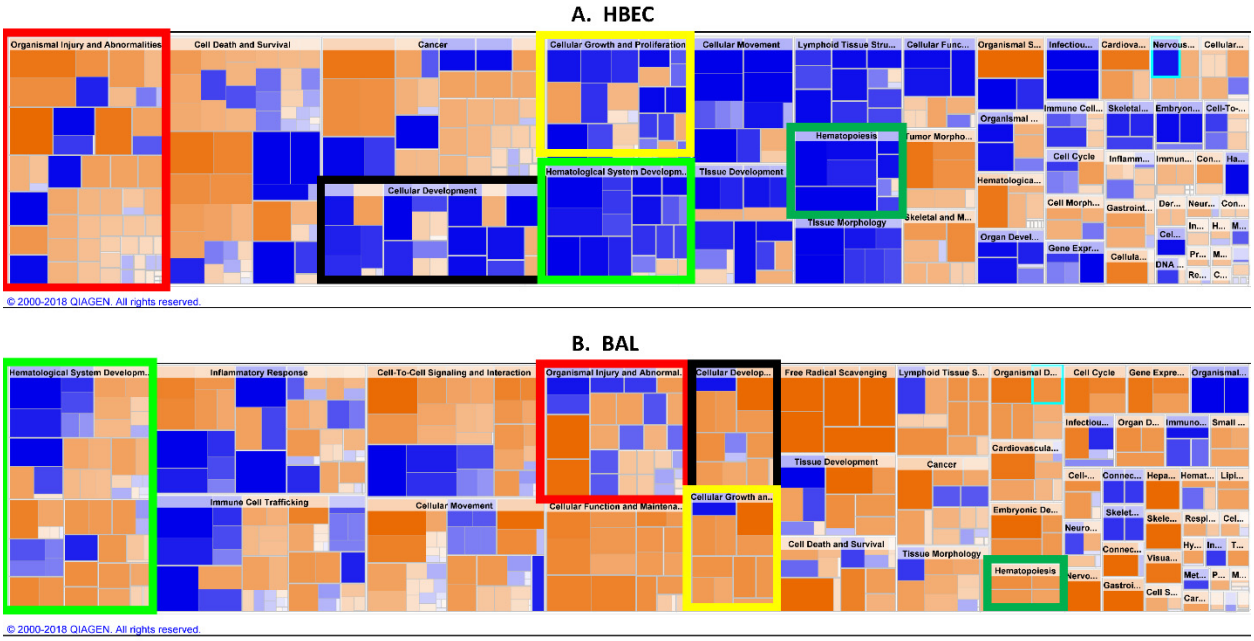


Figure 9. Comparison of activation state of diseases and biological function pathways in smokers during flight conditions for HBEC and BAL cells. Individual cells are sized according to magnitude of Z-score and colored according to direction and magnitude of Z-score. Blue represents suppression and orange represents activation. A. Diseases and Functions for HBEC cells. Large-scale suppression of noted pathways is evident. B. BAL cellular response is typified by activation of most cellular processes. Colored boxes represent disease/functions shared between the two cell types, identically-colored boxes represent the same disease or functional grouping in either cell type.

The largest effects among available contrasts, as determined by the R/timecourse Hotelling T^2 score, were related to the effects of smoking and gender (Table 6, Figure 10). Effects related to smokers during flight were much less, with T^2 scores typically under 40 for comparisons of smokers and nonsmokers at both flight and ground. In contrast, considering males and females separately when making contrasts produced increased T^2 scores. Using a T^2 cutoff of > 20 to assign differential expression based upon the Hotelling T^2 score distribution histogram (Figure 10), 48 genes were ruled significant DE in the FS v FN comparison, and 84 genes were ruled DE in the GS v GN comparison. Only three TCs were common to the two DE sets; TC01000004.hg.1 (LINC RNA), TC03000494.hg.1 (G-protein coupled receptor 15), and TC07003206.hg.1 (mRNA-like lincRNA), these were not considered in further analyses as they are likely due primarily to smoking due to their differential expression in the GSvGN DE list. The resulting purged list of 45 differentially expressed transcript clusters contained only 4 protein-coding genes; *IGLV2-33*,

OXER1, *TP53BP1*, and *HCAR3*. The remaining DE TCs mapped to snoRNA, snRNA, piRNA, lncRNA, and lincRNA genes lacking known roles in hypoxic responses. Additionally, one gene was downregulated during flight according to the FvG comparison (T^2= 28.06), *NACC2*, a transcriptional repressor that increases during hypoxia in endothelial progenitor cells (Wu TW et al. 2018), but its predictive ability is questionable due to high variability in expression.

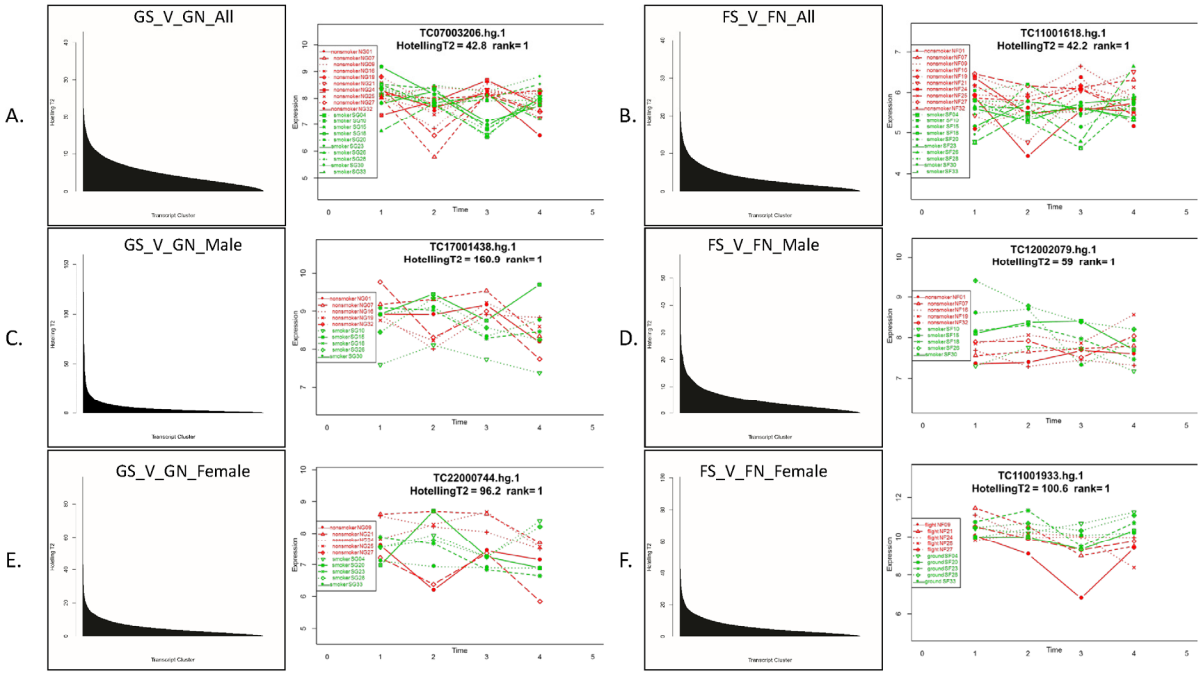


Figure 10. Hotelling T^2 score histogram and top transcript cluster for each contrast of blood-derived gene expression values. Comparing smokers (S) and nonsmokers (N) of each gender (F and M) by flight status produced results of differing significance according to Hotelling T^2 score. A. Comparison of all individuals during the ground phase produced top scores of less than 50, with the majority of genes scoring below 10. B. Comparison of all smokers and nonsmokers at the flight condition. The top regulated TC in this comparison changed most clearly at timepoint 3. C. Male ground smokers vs. male ground nonsmokers produced many higher-ranked TCs, with greater T^2 scores, than A and B. D. Male Flight Smokers vs. Male Ground Smokers. The top TC was suppressed in smokers over the course of the exposure and constant in nonsmokers. E. Female ground smokers vs. female ground nonsmokers produced higher-ranked TCs, with greater T^2 scores, than A and B. The top gene in this instance was quite different at timepoints 2 and 3. D. Female Flight Smokers vs. Female Ground Smokers. The top TC was activated in smokers over the course of the exposure.

To analyze the predicted impacts of the purged list of 45 differentially expressed genes (Supplementary Table 4), we extracted log fold change data from the R/limma comparison of smokers vs. nonsmokers at altitude (hours 1 and 5) minus smokers vs. nonsmokers at the ground state (hour 0). This comparison did not consider hour 6.5, but provided analysis of log fold change solely during the flight phase compared to the ground phase. The resulting data was submitted to IPA. As IPA's filtering method relies on a traditional p-value or FDR with confidence increasing as the figure nears 0, we calculated the inverse of the hoteling T^2 score ($1/T^2$) for each of the noted DE transcript clusters as a measure of predictive confidence. As

many of the transcript clusters on the list annotated to pseudogenes, ncRNA, and tRNAs, only 15 of the 45 transcript clusters submitted were considered analysis-ready. IPA analysis of the 15 analysis-ready genes resulted in only three significantly enriched canonical pathways; Interferon signaling (p= 0.0093), *ATM* signaling (p= 0.0257), and *Gαs* signaling (p= 0.0286). Z-scores detailing the direction of change could not be calculated for any of the identified pathways due to the scarcity of genes in the analysis; each of these canonical pathways was based upon the expression status of only one gene.

The canonical pathways identified suggest a hypoxic response. The Interferon signaling pathway was predicted based on the downregulation of Interferon Alpha Inducible Protein 6 (*IFI6*) at altitude. *IFI6* has been observed to increase during hypoxia (Fu et al. 2012), and its relative decline in smokers during flight indicates a potential inhibited response to mild hypoxia. The *ATM* signaling pathway was indicated by the downregulation of *TP53BP1* (*53BP1*), a hypoxia-responsive gene responsible for the phosphorylation and activation of *TP53*-induced transcription (Iwabuchi et al. 1998, Cuella-Martin et al. 2016) and phosphorylated by ATM (Lee et al. 2008). The Gαs signaling pathway was indicated by the downregulation of *HCAR3* (Hydroxycarboxylic acid receptor 3). HCAR3 activates ERK1/2 (Zhou et al. 2012), which in turn activates NFκB-mediated hypoxia responses (Osorio-Fuentealba et al. 2009). The downregulation of *HCAR3* at altitude in smokers may indicate inhibition in the hypoxic response in relation to nonsmokers. The canonical pathways indicated by IPA analysis may signify a general downregulation or delay in the hypoxic response of smokers to altitude.

Only one regulatory network was assembled from the differential expression list in IPA, including each of the three genes indicated in the three significant canonical pathways (Table 4, Figure 11). In this network, *TP53* plays a role as central regulator, in turn regulated by the suppression of *TP53BP1*. *TP53* then regulates *IFI6* via *ISG15* and regulates *HCAR3* through *CEBPA* (Figure 11). The observed downregulation of *TP53BP1* may be the result of an inhibition of the *TP53*-mediated hypoxia response in smokers, rather than a true suppression. Interestingly, *TP53* is overexpressed in non-small cell lung cancers (Xie et al. 2014), however, mutation in the TP53 protein also increases the propensity for tumor development (Aubrey et al. 2016). As the differential expression results of the blood analysis were largely insignificant, the effects of moderate hypoxia as noted in blood during and immediately after the hypoxic exposure are very small, even in the comparison between smokers and nonsmokers.

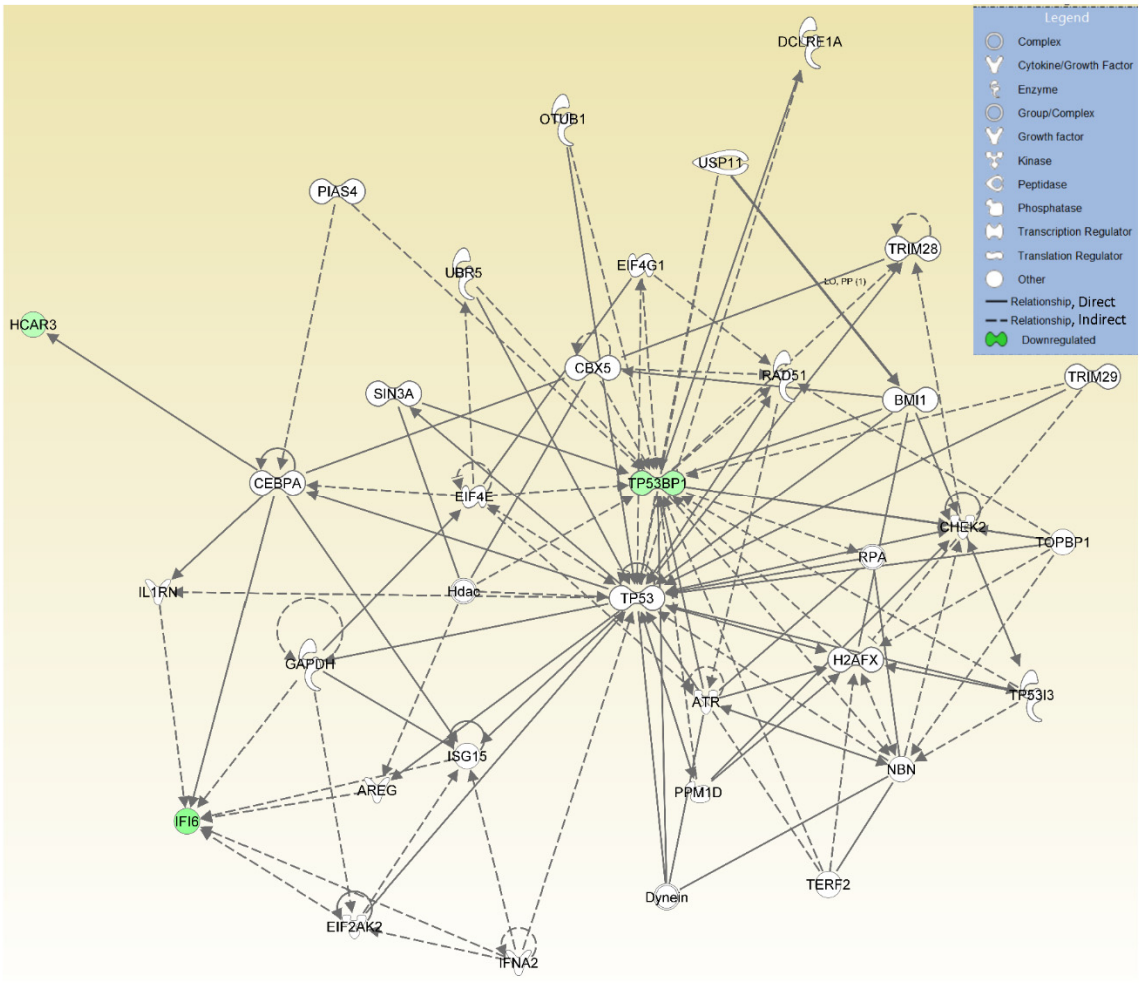


Figure 11. Top network identified in FS-FN comparison in blood samples. Samples from each timepoint over the course of the flight and ground exposures were analyzed using R/timecourse software. Genes with Hotelling T^2 scores exceeding 20 were declared differentially expressed. One network was identified based on the suppression of three genes (highlighted in green) in smokers during flight. The network identified centers on TP53 and TP53BP. Molecules highlighted in green are downregulated, white molecules have no direction-of-change information in the data set supplied to IPA.

Conclusions

The dangers of smoking are well recognized, and include predisposition to cancers, heart disease, atherosclerosis, COPD, and a host of additional maladies (United States Department of Health and Human Services 2014). The difficulty of physiological and molecular adaptation among smokers to altitude-induced hypoxia is also well-documented (Wu et al. 2012, Song et al. 2014, Daijo et al. 2016, Hussain and Tripathis 2018). Here, we presented evidence that smoking is a significant driver of gene expression in the very-mildly hypoxic environment of a hypobaric chamber simulating commercial aircraft travel. Differential responses of HBEC and BAL populations to flight between smokers and nonsmokers were observed that suggest an

inhibited hypoxic response in smokers, and which may result in enrichment of inflammatory, organismal injury, molecular transport, and cellular development responses in the alveolar cells (BAL), and an enrichment of cell death, cancer-related, and cellular growth responses in the bronchial epithelium (HBEC). In blood, the effect of altitude on gene expression in smokers vs. nonsmokers was very small; the alternate R/timecourse method was required to find DE biomarker candidates between smokers and nonsmokers during flight, with a heuristically-determined DE threshold. Regardless of the low threshold, the blood DE gene set may implicate a reduced hypoxic response in smokers during flight at 8,000 feet. The physiological responses observed in smoking and nonsmoking groups were in accordance with other studies (Nesthus et al. 1997, Wu et al. 2012, Song et al. 2014) involving altitude-related hypoxia. The main physiological differences between smokers and nonsmokers during altitude exposure were in subcutaneous blood O_2 saturation (similar between groups, but increasing over the course of exposure in smokers), heart rate (increasing in smokers over ground state), and exhaled CO_2 (slightly greater in smokers over the course of flight and ground exposures).

One of the goals of this study was to generate a list of useful blood biomarkers for the mild hypoxia induced by flight. The most applicable comparison for such a list, a direct comparison of flight and ground states among the entire population comprising both male and female smokers and nonsmokers, did not show any significant differential expression. This finding was potentially due to high inter-subject variability within the relatively small subject population. Further, comparisons among the blood samples yielded many fewer DE genes than did comparison between either cell type, likely due to the greater variability of blood samples.

The most applicable DE gene list obtained in this study for development of biomarkers is the 45-transcript cluster purged list derived from the blood comparison between smokers and nonsmokers in flight. The distinction between coding and non-coding genes is irrelevant in the search for RNA-based biomarkers, as any RNA species showing a consistent and predictable change may be used as a biomarker. In this study, one blood-based biomarker in the FvG comparison, *NACC2*, with the potential to diagnose flight-induced hypoxia was discovered, with a decrease in expression during flight (FvG, Table 6). However, the variability of expression likely will preclude its use as a predictive tool. Many additional potential biomarkers were found within the HBEC and BAL cellular populations, but the difficulty of obtaining cellular biopsies limit the utility of these biomarkers sets. Research on lung cells of smokers vs. nonsmokers still presents value beyond biomarker discovery, in enhancing understanding of the potential risks or biological effects of flight on these distinct groups. As with most such preliminary biomarker investigations, further research is necessary in a larger population.

The remaining contrast of most interest was that of smokers and nonsmokers in flight. As 15.5% of the U.S adult population are active cigarette smokers (US CDC, https://www.cdc.gov/tobacco/data_statistics/fact_sheets/adult_data/cig_smoking/index.html) , and smoking is known to exacerbate breathing difficulties, the effects of mild hypoxia on smokers was selected for examination in the current analysis. This study determined that

transcriptional changes induced to mitigate the mild hypoxia of the aircraft cabin were lacking in smokers, and in turn induced cellular responses that tended to drive increases in cellular damage responses and vascular growth in alveolar cells, while increases in cellular death and cancer pathways were observed in the bronchial epithelium. The long-term effects of continued activation of such processes is not known, but may be detrimental to proper lung function.

Supplementary Tables

Supplementary Table 1. Purged list of differentially expressed genes in smokers during flight in HBEC cells. LogFC = log2 fold change. AveExpr = average expression, t – limma moderated t-statistic with multiple testing correction, P.Value = p-value, adj.P.Val = Benjamini-Hochberg adjusted p-value (FDR), B = limma B-statistic (log-odds), HypoxiaGene = TRUE if this gene is hypoxia-related, FALSE if not. Present in BAL_List = this transcript cluster present in BAL DE list (Supplementary Table 2), ENSEMBL ID = ENSEMBL gene ID, Number of probes = number of individual probes making up transcript cluster. "0" in columns K-N = No Information Available.

https://www.faa.gov/data_research/research/med_humanfacs/oamtechreports/2020s/media/supplementary_table_1.xlsx

Figure 1Supplementary Table 1. Purged list of differentially expressed genes in smokers during flight in HBEC cells. LogFC = log2 fold change. AveExpr = average expression, t – limma moderated t-statistic with multiple testing correction, P.Value = p-value, a

Supplementary Table 2. Purged list of differentially expressed genes in smokers during flight in BAL cells. LogFC = log2 fold change. AveExpr = average expression, t = limma t-statistic with multiple-testing correction, P.Value = p-value, adj.P.Val = Benjamini-Hochberg adjusted p-value (FDR), B = limma B-statistic (log-odds), HypoxiaGene = TRUE if this gene is hypoxia-related, FALSE if not. ENSEMBL ID = ENSEBL gene ID, Number of probes = number of individual probes making up transcript cluster. "0" in columns J-M = No Information Available.

https://www.faa.gov/data_research/research/med_humanfacs/oamtechreports/2020s/media/supplementary_table_2.xlsx

Figure 2Supplementary Table 2. Purged list of differentially expressed genes in smokers during flight in BAL cells. LogFC = log2 fold change. AveExpr = average expression, t = limma t-statistic with multiple-testing correction, P.Value = p-value, adj.P.Val =

Supplementary Table 3. Significantly-induced canonical pathways in bal and hbec cells in smokers during flight.

https://www.faa.gov/data_research/research/med_humanfacs/oamtechreports/2020s/media/supplementary_table_3.xlsx

Figure 3Supplementary Table 3. Significantly-induced canonical pathways in bal and hbec cells in smokers during flight.

Supplementary Table 4. Purged list of differentially expressed genes in smokers during flight in blood samples. TranscriptID = transcript corresponding to transcript cluster. Multiple similarities (i.e., isoforms, etc.) separated by "///".

https://www.faa.gov/data_research/research/med_humanfacs/oamtechreports/2020s/media/supplementary_table_4.xlsx

Figure 4Supplementary Table 4. Purged list of differentially expressed genes in smokers during flight in blood samples. TranscriptID = transcript corresponding to transcript cluster. Multiple similarities (i.e., isoforms, etc.) separated by "///".

References

Ang SY, Uebersohn A, Spencer CI, Huang Y, Lee JE, Ge K, Bruneau BG. (2016) KMTD2 regulates specific programs in heart development via histone H3 lysine 4 di-methylation. *Development* 143(5): 810-821

Aubrey BJ, Strasser A, Kelly GL. (2016) Tumor-suppressor functions of the TP53 pathway. *Cold Spring Harbor Perspectives in Medicine* 6: a026062

Bencokova Z, Kaufmann M.R., Pires IM, Lecane PS, Giaccia AJ, Hammond EM. (2009) ATM Activation and Signaling under Hypoxic Conditions. *Molecular and Cellular Biology* 29(2): 526–537

Brugarolas J, Lei K, Hurley RL, Manning BD, Reiling JH, Hafen E, Witters LA, Ellisen LW, Kaelin Jr. WG. (2004) Regulation of mTOR function in response to hypoxia by REDD1 and the TSC1/TSC2 tumor suppressor complex. *Genes and Development* 18: 2893–2904

Burki NK, Tetenta SU. (2014) Inflammatory response to acute hypoxia in humans. *Pulmonary Pharmacology Therapeutics* 27(2): 208-211

Carvalho BS, Irizarry RA. (2010) A framework for oligonucleotide microarray preprocessing. *Bioinformatics* 26(19): 2363-2367

Chen M, Xiong F, Zhang L. (2013) Promoter methylation of EGR-1 site contributes to fetal hypoxia-mediated PKCe gene repression in the developing heart. *American Journal of Physiology - Regulatory Integrative Comparative Physiology* 304(9): R683-R689

Cuella-Martin R, Oliveira C, Lockstone HE, Snellenberg S, Grolmusova N, Chapman JR. (2016) 53BP1 Integrates DNA Repair and p53-Dependent Cell Fate Decisions via Distinct Mechanisms. *Molecular Cell* 64: 51–64

D'Ignazio L, Bandarra D, Rocha S. (2016) NF-kB and HIF crosstalk in immune responses. *FEBS Journal* 283(3): 413-424

Daijo H, Hoshino Y, Kai S, Suzuki K, Nishi K, Matsuo Y, Harada H, Hirota K. (2016) Cigarette smoke reversibly activates hypoxia-inducible factor 1 in a reactive oxygen species-dependent manner. *Scientific Reports* 6: 34424

Deng S, Yang Y, Han Y, Li X, Wang X, Li X, Zhang Z, Wang Y. (2012) UCP2 Inhibits ROS-Mediated Apoptosis in A549 under Hypoxic Conditions. *PLoS ONE* 7(1): e30714

Eales KL, Hollinshead KER, Tennant DA. (2016) Hypoxia and metabolic adaptation of cancer cells. *Nature:Oncogenesis* 5: 190

Fandrey J, Gorr TA, Gassmann M. (2006) Regulating cellular oxygen sensing by hydroxylation. *Cardiovascular Research* 71(4): 642-651

Fang H-Y, Hughes R, Murcoch C, Coffelt SB, Biswas SK, Harris AL, Johnson RS, Imityaz HZ, Simon MC, Fredlund E, Greten FR, Rius J, Lewis CE. (2009) Hypoxia-inducible factors 1 and 2 are important transcriptional effectors in primary macrophages experiencing hypoxia. *Blood* 114: 844-859

Fu L, Chen L, Yang J, Ye T, Chen Y and Fang J. (2012) HIF-1α-induced histone demethylase JMJD2B contributes to the malignant phenotype of colorectal cancer cells via an epigenetic mechanism. *Carcinogenesis* 33(9): 1664–1673

Goodin JL, Pizarro-Matos JM, Prasad BM, Seiter TJ, Waever CR, Muza SR, Beidleman BA, Wood JC. (2013) Evaluating the molecular basis for acute mountain sickness: hypoxia response gene expression patterns in warfighters and murine populations. *Military Medicine* 178(11): 1256-1263

Heron M, Grutters JC, ten Dam-Molenkamp KM, Hijdra D, van Heugten-Roeling A, Claessen AME, Ruven HJT, van den Bosch. (2012) Bronchoalveolar lavage cell pattern from healthy human lung. *Clinical and Experimental Immunology* 167(3): 523-531

Hu CJ, Iyer S, Sataur A, Covello KL, Chodosh LA, Simon MC. (2006) Differential Regulation of the Transcriptional Activities of Hypoxia-Inducible Factor 1 Alpha (HIF-1α) and HIF-2α in Stem Cells. *Molecular and Cellular Biology* 26(9): 3514-3526

Hussain S and Tripathi V. (2018) Smoking under hypoxic conditions: a potent environmental risk factor for inflammatory and autoimmune diseases. *Military Medical Research* 5:11

Huxtable AG, Smith SMC, Peterson TJ, Watters JJ, Mitchell GS. (2015) Intermittent Hypoxia-Induced Spinal Inflammation Impairs Respiratory Motor Plasticity by a Spinal p38 MAP Kinase-Dependent Mechanism. *The Journal of Neuroscience* 35(17): 6871-6880

Iwabuchi K, Li B, Massa HF, Trask BJ, Date T, and Fields S. (1998) Stimulation of p53-mediated Transcriptional Activation by the p53-binding Proteins, 53BP1 and 53BP2. *Journal of Biological Chemistry* 273(40): 26061–26068

Jacobs MD, Harrison SC. (1998) Structure of an IkBa/NF-kB complex. *Cell* 95: 749-758

Jantsch J, Weise M, Schodel J, Castiglione K, Glasner J, Kolbe S, Mole D, Schliecher U, Eckardt KW, Hensel M, Lang R, Bogdan C, Schnare M, Willam C. (2011) Toll-like receptor activation and hypoxia use distinct signaling pathways to stabilize hypoxia-inducible factor 1α (HIF1A) and result in differential HIF1A-dependent gene expression. *Journal of Leukocyte Biology* 90(3): 551-562

Jia C, Kong X, Koltes JE, Gou X, Yang S, Yan D, Lu S, Wei Z. (2016) Gene co-expression network analysis unraveling transcriptional regulation of high-altitude adaptation of Tibetan pig. *PLoS One* 11(2): e0168161

Karimi R, Tornling G, Grunewald J, Eklund A, Sköld CM. (2012) Cell recovery in bronchoalveolar lavage fluid in smokers is dependent on cumulative smoking history. *PLoS One* 7(3): e34232

Kauffmann A, Gentleman R, Huber W. (2009) arrayQualityMetrics–a bioconductor package for quality assessment of microarray data. *Bioinformatics* 25(3): 415–416

Kenney JW, Poole RL, Adoff MD, Logue SF, Gould TJ. (2012) Learning and Nicotine Interact to Increase CREB Phosphorylation at the jnk1 Promoter in the Hippocampus. *PLoS One* 7(6): e39939

Kilic-Eren M, Boylu T and Tabor V. (2013) Targeting PI3K/Akt represses Hypoxia inducible factor-1α activation and sensitizes Rhabdomyosarcoma and Ewing's sarcoma cells for apoptosis. *Cancer Cell International* 13: 36

Kim JY, Ahn HJ, Ryu JH, Suk K, and Park JH. (2004) BH3-only Protein Noxa Is a Mediator of Hypoxic Cell Death Induced by Hypoxia-inducible Factor 1. *Journal of Experimental Medicine* 199(1): 113–123

Kim SY, Choi YJ, Joung SM, Lee BH, Jung YS and Lee JY. (2009) Hypoxic stress up-regulates the expression of Toll-like receptor 4 in macrophages via hypoxia-inducible factor. *Immunology* 129: 516–524

Kong X, Lin Z, Liang D, Fath D, Sang N, Caro J. (2006) Histone deacetylase inhibitors induce VHL and ubiquitin-dependent degradation of hypoxia-inducible factor 1a. *Molecular and Cellular Biology* 26(6): 2019-2028

Koong AC, Chen EY, Giarrca AJ. (1994) Hypoxia causes the activation of nuclear factor kappa B through the phosphorylation of I kappa B alpha on tyrosine residues. *Cancer Research* 54(6): 1425-1430

Langer HF, Chavakis T. (2009) Leukocyte-endothelial interactions in inflammation. *Journal of Cellular and Molecular Medicine* 13(7): 1211-1220

Lee H, Kwak HJ, Cho IT, Park SH and Lee CH. (2008) S1219 residue of 53BP1 is phosphorylated by ATM kinase upon DNA damage and required for proper execution of DNA damage response. *Biochemical and Biophysical Communications* 378(1): 32-36

Leonard MO, Howell K, Madden SF, Costello CM, Higgins DG, Taylor CT and McLoughlin P. (2008) Hypoxia Selectively Activates the CREB Family of Transcription Factors in the In Vivo Lung. *American Journal of Respiratory and Critical Care Medicine* 178: 977–983

Lima WF, Wu H, Nichols JG, Sun H, Murray HM, Crooke ST. (2009) Binding and Cleavage Specificities of Human Argonaute2. *The Journal of Biological Chemistry* 284(38): 26017-26028

Liu B, Chen J, Zhang L, Gao Y, Cui J, Zhang E, Xu G, Liang Y, Liang Y, Wang J and Gao Y. (2017) IL-10 dysregulation in acute mountain sickness revealed by transcriptome analysis. *Frontiers in Immunology* 8: 628

Liu J, Narasimhan P, Lee YS, Song YS, Endo H, Yu F and Chan PH. (2006) Mild hypoxia promotes survival and proliferation of SOD2-deficient astrocytes via c-Myc activation. *Journal of Neuroscience* 26(16): 4329-4337

Liu Y, Nie H, Zhang K, Ma D, Yang G, Zheng Z, Liu K, Yu B, Zhai C, Yang S. (2014) A feedback regulatory loop between HIA-1a and miR-21 in response to hypoxia in cardiomyocytes. *FEBS Letters* 588: 3137-3146

Mace TA, Collins AL, Wojcik SE, Croce CM, Lesinski GB, Boolston M. (2013) Hypoxia induces the overexpression of MicroRNA-21 in pancreatic cancer cells. *Journal of Surgical Research* 184(2): 855-860

Marin MC and Marques MM. (2016) Novel role of p73 as a regulator of developmentak angiogenesis: implication for cancer therapy. *Molecular and Cellular Oncology* 3(1): e1019973

McKeown SR. (2014) Defining normoxia, physoxia and hypoxia in tumours-implications for treatment response. *British Journal of Radiology* 87: 20130676

Minet E, Arnould T, Michel G, Roland I, Mottet D, Raes M, Remacle J, Michiels C. (2000) ERK activation upon hypoxia: involvement in HIF-1 activation. *FEBS Letters* 468(1): 53-58

Morfoisse F, Renaud E, Hantelys F, Prats A-C, Garmy-Susini B. (2015) Role of hypoxia and vascular endothelial growth factors in lymphangiogenesis. *Molecular and Cellular Oncology* 2(4): e1024821

Muhm JM, Rock PB, McMullin DL, Jones SP, Lu LL, Eilers KD, Space DR, McMullen A. (2007) Effect of aircraft-cabin altitude on passenger discomfort. *New England Journal of Medicine* 357: 18-27

Muller JM, Krauss B, Kaltschmidt C, Baeurrle PA, Rupec RA. (1997) Hypoxia induces c-fos transcription via a mitogen-activated protein kinase-dependent pathway. *Journal of Biological Chemistry* 272: 23435-23439

Muz B, de la Puente P, Azab F, Azab AK. (2015) The role of hypoxia in cancer progression, angiogenesis, metastasis, and resistance to therapy. *Hypoxia (Auckland)* 3: 83-92

Nesthus TE, Garner RP, Mills SH, Wise RA. (1997) Effects of simulated general aviation hypoxia on smokers and nonsmokers. *Office of Aviation Medicine* March 1997

Nie M1, Balda MS, Matter K. (2012) Stress- and Rho-activated ZO-1-associated nucleic acid binding protein binding to p21 mRNA mediates stabilization, translation, and cell survival. *Proceedings of the National Academy of Sciences USA* 109(27): 10897-10902

Nillson GE, Winberg S. (1993) Changes in the brain levels of GABA and related amino acids in anoxic shore crab (*Carcinus maenas*). *American Journal of Physiology* 264(4pt2): 733-777

Olcina MM, Grand RJ, Hammond EM. (2014) ATM activation in hypoxia - causes and consequences. *Molecular and Cellular Oncology* 1(1): e29903

Osorio-Fuenteabla C, Valdes JA, Riquelme D, Hidalgo J, Hidalgo C, Carrasco MA. (2009) Hypoxia stimulates via separate pathways ERK phosphorylation and NF-kB activation in skeletal muscle cells in primary culture. *Journal of Applied Physiology* 106: 1301-1310

Palazon A, Goldrath AW, Nizet V, Johnson RS. (2014) HIF transcription factors, inflammation, and immunity. *Immunity* 41(4): 518-528

Parviz M, Vogel K, Gibson KM, Pearl PL. (2014) Disorders of GABA metabolism: SSADH and GABA-transaminase deficiencies. *Journal of Pediatric Epilepsy* 3(4): 217-227.

Peacock AJ. (1998) Oxygen at high altitude. *British Medical Journal* 317: 1063-1067

Pimentel-Coelho PM, Michaud JP, Rivest S. (2013) Evidence for a gender-specific protective role of innate immune receptors in a model of perinatal brain injury. *Journal of Neuroscience* 33(28): 11556-11572

Rajakumar PS, Bhat BV, Sridhar MG, Balachander J, Konar BC, Narayanan P, Chetan G. (2008) Cardiac enzyme levels in myocardial dysfunction in newborns with perinatal asphyxia. *Indian Journal of Pediatrics* 75(12): 1223-1225

Richard DE, Berra E, Gothie E, le Roux D, Pouyssegur J. (1999) p42/p44 Mitogen-activated Protein Kinases Phosphorylate Hypoxia-inducible Factor 1a (HIF-1a) and Enhance the Transcriptional Activity of HIF-1. *Journal of Biological Chemistry* 274(46): 32631–32637

Ritchie ME, Phipson B, Wu D, Hu Y, Law CW, Shi W, Smyth GK. (2015) limma powers differential expression analyses for RNA-sequencing and microarray studies. *Nucleic Acids Research* 43(7): e47

Roman J, Rangasamy T, Guo J, Sugunan S, Meednu N, Packirisamy G, Shimoda LA, Golding A, Semenza G, Georas SN. (2010) T-Cell Activation under Hypoxic Conditions Enhances IFN-γ Secretion. *American Journal of Respiratory Cellular and Molecular Biology* 42(1): 123–128

Royds JA, Dower SK, Qwarnstrom EE, Lewis CE. (1998) Response of tumour cells to hypoxia: role of p53 and NFkB. *Molecular Pathology* 51(2): 55-61

Sabapathy K. (2016) p73: a positive or negative regulator of angiogenesis, or both? *Molecular and Cellular Biology* 36(6): 848-854

Sagone AL, Lawrence T, Balcerzak SP. (1973) Effect of smoking on tissue oxygen supply. *Blood* 41(6): 845-851

Samuels MP. (2004) The effects of flight and altitude. *Archives of Disease in Childhood* 89: 448-455

Sarkar S, Banerjee PK, Selvamurthy W. (2003) High altitude hypoxia: an intricate interplay of oxygen responsinve macroevents and micromolecules. *Molecular Cellular Biochemistry* 253: 287-395

Schmidt KM, Hellerbrand C, Ruemmele P, Michalski CW, Kong B, Kroemer A, Hackl C, Schlitt HJ, Geissler EK, Lang SA. (2017) Inhibition of mTORC2 component RICTOR impairs tumor growth in pancreatic cancer models. *Oncotarget* 8(15): 24491-24505

Semenza G, Wang GL. (1992) A nuclear factor induced by hypoxia via de novo protein synthesis binds to human erythropoietin gene enhancer at a site required for transcriptional activation. *Molecular and Cellular Biology* 12(12): 5447-5454

Semenza GL. (2011) Regulation of Metabolism by hypoxia-inducible factor 1. *Cold Spring Harbor Symposia on Quantitative Biology* Volume LXXVI: 347-353

Shen J, Xia W, Khotskaya, YB, Huo L, Nakanishi K, Lim S-O, Du Y, Wang Y, Chang W-C, Chen C-H, Hsu JL, Wu Y, Lam YC, James BP, Liu X, Patel DW, Hung M-C. (2013) EGFR modulates

microRNA maturation in response to hypoxia through phosphorylation of AGO2. *Nature* 497(7449): 383–387

Shin DH, Li SH, Yang SW, Lee BL, Lee MK, Park JW. (2009) Inhibitor of nuclear factor-kappaB alpha derepresses hypoxia-inducible factor-1 during moderate hypoxia by sequestering factor inhibiting hypoxia-inducible factor from hypoxia-inducible factor 1alpha. *FEBS Journal* 276(13): 3470-3480

Shrestha N, Bahnan W, Wiley DJ, Barber G, Fields KA, Schesser K. (2012) Eukaryotic initiation factor 2 (eIF2) signaling regulates proinflammatory cytokine expression and bacterial invasion. *Journal of Biological Chemistry* 287(34): 28738-28744

Simon MC, Liu L, Barnhart BC, Young RM. (2008) Hypoxia-Induced Signaling in the Cardiovascular System. *Annual Reviews in Physiology* 70: 51-71

Song P, Zhang JH, Qin J, Gao XB, Yu J, Tang XG, Tang CF, Huang L. (2014) Smoking is associated with the incidence of AMS: a large-sample cohort study. *Military Medical Research.* 1: 16

Suzuki A, Kusakai I, Shimojo Y, Chen J, Ogura T, Kobayasji M, Esumi H. (2006) Involvement of transforming growth factor-B1 signaling in hypoxia-induced tolerance to glucose starvation. *Journal of Biological Chemistry* 280(36): 31557-31563

Tai YC. (2007) *timecourse: Statistical Analysis for Developmental Microarray Time Course Data*. R package version 1.52.0, http://www.bioconductor.org

Teteloshvili N, Smigielaska-Czepiel K, Yuan Y, Seitz A, de Jong D, Rutgers B, Jellema P, van der Lei RJ, Slezak-Prochazka I, Brouwer E, Boots AMH, Kroesen B-J, van den Berg A, Kluiver J. (2017) Argonaute 2 immunoprecipitation revealed large tumor suppressor kinase 1 as a novel proapoptotic target of miR-21 in T cells. *FEBS Journal* 284: 555-567

Thompson AB, Mueller MB, Heires AJ, Buhling TL, Daughton D, Yancey SW, Sykes RS, Rennard SI. (1992) Aerosolized beclamethasone in chronic bronchitis: Improved pulmonary function and diminished airway inflammation. *American Review of Respiratory Disease* 146(2): 389-395

Trollman R, Rehrauer H, Schneider C, Krischke G, Huemmler N, Keller S, Rascher W, Gassmann M. (2010) Late-gestational systemic hypoxia leads to a similar early gene response in mouse placenta and developing brain. *American Journal of Physiology: Regulatory Integrative and Comparative Physiology* 299: R1489-R1499

U.S. Department of Health and Human Services. (2014) *The Health Consequences of Smoking: 50 Years of Progress. A Report of the Surgeon General.* Atlanta, GA: U.S. Department of Health and Human Services, Centers for Disease Control and Prevention, National Center for Chronic Disease Prevention and Health Promotion, Office on Smoking and Health.

Van Uden P, Niall KS and Rocha S. (2008) Regulation of hypoxia-inducible factor-1α by NF-KB. *Biochemistry Journal* 412: 477–484

Villunger A1, Michalak EM, Coultas L, Müllauer F, Böck G, Ausserlechner MJ, Adams JM, Strasser A. (2003) p53- and drug-induced apoptotic responses mediated by BH3-only proteins puma and noxa. *Science* 302(5647): 1036-1038

Wang Y, Liu T, Yang N, Xu S, Li X, Wang D. (2016) Hypoxia and macrophages promote glioblastoma invasion by the CCL4-CCR5 axis. *Oncology Reports* 36(6): 3522-3528

Wu G, Xu G, Chen DW, Gao WX, Xiong JQ, Shen HY, Gao YQ. (2018) Hypoxia Exacerbates Inflammatory Acute Lung Injury via the Toll-Like Receptor 4 Signaling Pathway. *Frontiers in Immunology* 9: 1667

Wu TW, Liu CC, Hung CL, Yen CH, Wu YJ, Wang LY, Yeh HI. (2018) Genetic profiling of young and aged endothelial progenitor cells in hypoxia. *PLoS One* 13(4): e0196572

Wu TY, Ding SQ, Liu JL, Jia JH, Chai ZC, Dai RC, Zhao JZ, Tang QD, Kayser B. (2012) Smoking, acute mountain sickness and altitude acclimatisation: a cohort study. *Thorax* 67(10): 914-919

Wu W, Dave N, Yu G, Strollo PJ, Kovkarove-Naumovski E, Ryter SW, Reeves SR, Dayyat E, Wang Y, Choi AMK, Gozal D, Kaminski N. (2008) Network analysis of temporal effects of intermittent and sustained hypoxia on rat lungs. *Physiological Genomics* 36: 24-34

Yamaguchi J, Tanaka T, Eto N, Nagaku M. (2015) Inflammation and hypoxia linked to renal injury by CCAAT/enhancer-binding protein δ. *Kidney International* 88: 262-275

Yan Y, Sji Y, Wang C, Guo P, Wang J, Zhang C-Y, Zhang C. (2015) Influence of a high-altitude hypoxic environment on human plasma microRNA profiles. *Scientific Reports* 5: 15156

Zhang C, Jones JT, Chand HS, Wathelet MG, Evans CM, Dickey B, Xiang J, Mebratu YA, Tesfaigzi Y. (2018) Noxa/HSP27 complex delays degradation of ubiquitylated IkBα in airway epithelial cells to reduce pulmonary inflammation. *Mucosal Immunology* 11(3): 741-751.

Zhang N, Walker MK. (2007) Crosstalk between the aryl hydrocarbon receprot and hypoxia on the constitutive expression of cytochrome P4501A1 mRNA. *Cardiovascular Toxicology* 7(4): 282-290

Zhou Q, Li G, Deng XY, He XB, Chen LJ, Wu C, Shi Y, Wu KP, Mei LJ, Lu JX, Zhou NM. (2012) Activated human hydroxy-carboxylic acid receptor-3 signals to MAP kinase cascades via the PLC-dependent PKC and MMP-mediated EGFR pathways. *British Journal of Pharmacology* 166(6): 1756-1773.