

1 **Neonatal hyperoxia enhances age-dependent expression**
2 **of SARS-CoV-2 receptors in mice**

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34 **ABSTRACT**

35 The severity of COVID-19 lung disease is higher in the elderly and people with pre-existing co-
36 morbidity. People who were born preterm may be at greater risk for COVID-19 because their early
37 exposure to oxygen at birth increases their risk of being hospitalized when infected with RSV and
38 other respiratory viruses. Our prior studies in mice showed how high levels of oxygen (hyperoxia)
39 between postnatal days 0-4 increases the severity of influenza A virus infections by reducing the
40 number of alveolar epithelial type 2 (AT2) cells. Because AT2 cells express the SARS-CoV-2
41 receptors angiotensin converting enzyme (ACE2) and transmembrane protease/serine subfamily
42 member 2 (TMPRSS2), we expected their expression would decline as AT2 cells were depleted by
43 hyperoxia. Instead, we made the surprising discovery that expression of *Ace2* and *Tmprss2* mRNA
44 increases as mice age and is accelerated by exposing mice to neonatal hyperoxia. ACE2 is primarily
45 expressed at birth by airway Club cells and becomes detectable in AT2 cells by one year of life.
46 Neonatal hyperoxia increases ACE2 expression in Club cells and makes it detectable in 2-month-old
47 AT2 cells. This early and increased expression of SARS-CoV-2 receptors was not seen in adult mice
48 who had been administered the mitochondrial superoxide scavenger mitoTEMPO during hyperoxia.
49 Our finding that early life insults such as hyperoxia enhances the age-dependent expression of SARS-
50 CoV-2 receptors in the respiratory epithelium helps explain why COVID-19 lung disease is greater in
51 the elderly and people with pre-existing co-morbidities.

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55 **Key Words:** Angiotensin Converting Enzyme 2, COVID-19, Hyperoxia, Mice, Transmembrane
56 protease/serine subfamily member 2

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58 INTRODUCTION

59 COVID-19 is an infectious disease of the lung caused by the severe acute respiratory
60 syndrome coronavirus (SARS-CoV-2). As of July 2020, the World Health Organization reported this
61 virus has infected more than 10 million people worldwide and killed approximately 500,000 people
62 (<https://covid19.who.int>). Common symptoms include fever, cough, fatigue, shortness of breath, and
63 loss of olfactory or gustatory function. While the majority of cases are mild, some people progress into
64 severe acute respiratory distress syndrome, multi-organ failure, thrombosis, and septic shock. The
65 severity of disease and mortality is highest among the elderly and people who have pre-existing lung
66 or heart disease. There is growing evidence that asymptomatic children and young adults with
67 COVID-19 may be at risk for heart disease, inflammatory vascular disease, and stroke ¹. People who
68 were born preterm may be at great risk for COVID-19 because they are already at risk for
69 hospitalization following infection with RSV, rhinovirus, human bocavirus, metapneumovirus, and
70 parainfluenza viruses ². They may also develop pulmonary vascular disease and heart failure ^{3,4},
71 autism-like disorders ^{5,6}, and retinopathy ⁷ that puts them at further risk for COVID-19. Identifying
72 mechanisms that drive susceptibility to pandemic viral infections like SARS-CoV-2 is therefore of
73 great concern to susceptible individuals and their families.

74 The severity of COVID-19 is likely to be related to age-related changes in SARS-CoV-2
75 receptors and how the immune system responds to infection ¹. Emerging evidence indicates high-risk
76 individuals with SARS-CoV-2 have high rates of alveolar epithelial type 2 (AT2) cell infection,
77 suggesting disease severity may be related to higher alveolar expression of the SARS-CoV-2 receptor
78 angiotensin converting enzyme (ACE2) and its co-receptor transmembrane protease/serine subfamily
79 member 2 (TMPRSS2) ^{8,9}. In fact, a recent meta-analysis of 700 people with predicted
80 COVID-19 co-morbidities found that their lungs expressed high levels *Ace2* mRNA ¹⁰. ACE2 is a zinc
81 containing metalloprotease present at the surface of cells in the lung, heart, intestines, kidneys, and
82 brain. It lowers blood pressure by catalyzing the hydrolysis of the vasoconstrictive molecule
83 angiotensin II to angiotensin (1-7). ACE2 co-precipitates with transmembrane protease/serine
84 subfamily member 2 (TMPRSS2) which hydrolyzes the S protein on coronaviruses, thus enabling viral

85 entry into infected cells^{9,11}. Higher expression of these proteins in AT2 cells would theoretically lead
86 to higher rates of infection in the distal lung. Infected AT2 cells produce inflammatory mediators that
87 could contribute to a lethal cytokine storm^{12,13}. They may also die. Loss of AT2 cells below a critical
88 threshold could compromise alveolar homeostasis because they produce surfactant and serve as
89 adult stem cells for the alveolar epithelium¹⁴. In fact, high rates of AT2 infection have been seen in
90 people who have succumbed to H5N1, a highly pathogenic avian strain of influenza A virus¹⁵⁻¹⁷. But
91 whether aging or pre-existing lung co-morbidities like preterm birth enhance the severity of respiratory
92 viral infections via changing expression of viral receptors is not yet known.

93 Since preterm infants are exposed too soon to oxygen, we have been using mice to
94 understand how high levels of oxygen at birth increases the severity of influenza A virus infection in
95 adults. We previously reported how adult mice exposed to hyperoxia (100% oxygen) between
96 postnatal days 0-4 develop persistent inflammation and fibrotic lung disease when infected with
97 influenza A viruses HKx31 (H3N2) or PR8 (H1N1)^{18,19}. Neonatal hyperoxia does not enhance primary
98 infection²⁰ or clearance²¹ of the virus. Instead, it reduced the number of adult AT2 cells by ~50%,
99 thus lowering the number available to maintain alveolar homeostasis and epithelial regeneration after
100 infection²². Because neonatal hyperoxia reduces the number of AT2 cells, we predicted it would
101 reduce the alveolar expression of ACE2 and TMPRSS2 in the lung. Instead, we made the surprising
102 discovery that expression of ACE2 and TMPRSS2 increases as mice age and this age-dependent
103 expression can be enhanced by early exposure to hyperoxia. Our findings in mice suggest temporal
104 and spatial changes in expression of SARS-CoV-2 receptors may contribute to the increased severity
105 of COVID-19 seen in the elderly and people with pre-existing co-morbidities, including those born
106 preterm.

107

108 RESULTS

109 *ACE2 is initially expressed by Club cells and then by AT2 cells as mice age.* The localization
110 of ACE2 was examined in the lungs of mice between PND4 and 2 years of age by
111 immunohistochemistry so as to better understand the temporal spatial pattern of its expression. ACE2
112 was primarily detected in airway epithelial cells with minimal staining seen in the alveolar space
113 (**Figure 1a**). The intensity of ACE2 staining increased steadily in the airway epithelium throughout the
114 life of the mouse. A rare ACE2-positive alveolar cells (arrows) was first observed on PND7 and then
115 steadily increased in number between 6 and 24 months of age. Western blotting for ACE2 confirmed
116 that the abundance of ACE2 protein became progressively enriched in the whole lungs of 12- and 24-
117 month-old mice relative to those of mice harvested at 2 months of age (**Figure 1b**). ACE2 mRNA
118 levels were similarly increased in the whole lungs of 24-month-old mice than in those of mice
119 harvested at 2 months of age (**Figure 1c**).

120 Co-staining with antibodies for ACE2 and the Club cell marker secretoglobin1a1 (Scgb1a1)
121 showed extensive co-localization along the airways at both 2 and 12 months of age (**Figure 2a**), but
122 the intensity of ACE2 staining was significantly higher at 12 months of age than at 2 months of age
123 (**Figure 2b**). Co-staining for ACE2 and the AT2 cell marker proSP-C revealed that the vast majority of
124 ACE2+ cells in the alveoli were AT2 cells (**Figure 2c**). Approximately 20% of proSP-C+ AT2 cells
125 expressed ACE2 at 2 months while 80% of proSP-C+ AT2 cells expressed it at 12 months (**Figure**
126 **2d**). These findings reveal that ACE2 is primarily expressed by the airway Club cells of young adult
127 mice but becomes increasingly expressed by AT2 cells as mice age.

128
129 *Neonatal hyperoxia enhances the age-dependent changes in ACE2 expression.* We
130 previously showed that adult mice exposed to 100% oxygen between PND0-4 (**Figure 3a**) have fewer
131 AT2 cells than mice exposed to room air²³ and thus expected ACE2 expression to be lower in the
132 lungs of mice exposed to neonatal hyperoxia than in those of controls. It was therefore surprising to
133 find that the levels of ACE2 protein were higher in the lungs of 2-month-old mice that were exposed to
134 neonatal hyperoxia than in age-matched control lungs (**Figure 3b**). The levels of Ace2 mRNA were

135 also increased in the lungs of neonatal hyperoxia-exposed mice at 2 months of age and remained
136 higher than in the lungs of age-matched controls at 6 and 12 months of age (**Figure 3c**). To determine
137 the amount of oxygen needed to stimulate the expression of *Ace2*, the lungs of 2-month-old mice
138 exposed to 0, 40, 60 or 80% oxygen from PND0-4 were examined by qRT-PCR (**Figure 3d**). While
139 40% oxygen was not sufficient to induce *Ace2* mRNA, the levels of *Ace2* expression was significantly
140 higher in mice exposed to 60% and 80% oxygen relative to controls. Exposing mice to a low chronic
141 dose of oxygen (40% for 8 days) that does not alter alveolar development²⁴ also failed to increase
142 *Ace2* levels relative to controls (data not shown). Because 40% oxygen for 8 days is higher
143 cumulative dose of oxygen than 60% for 4 days, these findings suggest that oxygen alone may not be
144 stimulating *Ace2* expression.

145 Immunohistochemistry was used to further understand how hyperoxia affected ACE2
146 expression in the adult lung. While neonatal hyperoxia increased intensity of ACE2 staining in the
147 airway, it most obviously increased the number of alveolar cells with detectable ACE2 (**Figure 4a**).
148 When quantified, neonatal hyperoxia increased the number of alveolar cells expressing ACE2 by
149 approximately 50% at 2, 6 and 12 months of age (**Figure 4b**). The increased alveolar expression
150 seen at 2 months of age was primarily attributed to increased expression by proSP-C+ AT2 cells;
151 however, this difference resolved at 6 and 12 months of age as more AT2 cells in control lungs began
152 to express ACE2 (**Figure 4c**).

153
154 *Anti-oxidants block oxygen-dependent changes in ACE2 expression.* Prior studies by us and other
155 investigators showed that administering the mitochondrial superoxide scavenger mitoTEMPO to mice
156 during exposure to hyperoxia (**Figure 5a**) prevents the alveolar simplification and cardiovascular
157 disease observed when these mice reach adulthood²⁵⁻²⁷. qRT-PCR revealed administering
158 mitoTEMPO during hyperoxia blunted the oxygen-dependent increase in *Ace2* mRNA seen in 2-
159 month-old mice (**Figure 5a, b**). Immunohistochemistry confirmed mitoTEMPO reduced the number of
160 AT2 cells with detectable levels of ACE2 protein (**Figure 5c, d**). It also reduced the intensity of ACE2
161 staining in airway Club cells (**Figure 5e, f**). Interestingly, while mitoTEMPO did not affect ACE2

162 staining in control mice, it reduced the numbers of alveolar ACE2+ cells in the lungs of hyperoxia-
163 exposed mice lower than controls.

164

165 *Neonatal hyperoxia stimulates age-dependent changes in TMPRSS2.* TMPRSS2 is an
166 endoprotease expressed by respiratory epithelial cells that facilitates viral entry of coronaviruses into
167 epithelial cells⁹. The levels of *Tmprss2* mRNA and protein were examined in the lungs of 2-, 12- and
168 18-month-old mice that were exposed to neonatal hyperoxia and room air from PND0-4 by qRT-PCR
169 and western blotting. *Tmprss2* mRNA was readily detected in the lungs of 2-month-old mice, and
170 increased ~5-fold at 12 months and ~8-fold at 18 months (**Figure 6a**). Neonatal hyperoxia further
171 increased *Tmprss2* expression by ~50% at each time-point examined. Western blotting similarly
172 showed that the levels of TMPRSS2 protein were higher in the whole lung lysates of mice exposed to
173 neonatal hyperoxia than in those of control mice (**Figure 6b**). As observed for *Ace2* expression,
174 exposure to ≥ 60% oxygen from PND4-0 was required to significantly increase the levels of *Tmprss2*
175 mRNA in the lungs of mice at 2 months of age (**Figure 6c**). Exposure to 40% oxygen from PND0-8
176 also failed to change *Tmprss2* expression in adult mice (data not shown) while the administration of
177 mitoTEMPO to mice during exposure blunted the effects of neonatal hyperoxia on *Tmprss2* mRNA
178 (**Figure 6d**). Together, these findings suggest age and neonatal hyperoxia have similar effects on
179 increasing TMPRSS2 as they do for ACE2.

180

181 **DISCUSSION**

182 The COVID-19 outbreak was first detected in the Chinese city of Wuhan in 2019 and has since
183 expanded rapidly to become one of the worst pandemics to ever challenge the modern world. While
184 people of all ages are susceptible to infection, the severity of disease is worse in people who are
185 elderly or who have pre-existing health conditions including COPD, diabetes, hypertension, and
186 cancer²⁸. Those with multiple co-morbidities have a higher rate of mortality. People born preterm may
187 also be at great risk for COVID-19 because they often suffer from multiple co-morbidities due, in part,
188 to their lungs being exposed to oxygen too soon or to super-physiological concentrations used to
189 maintain appropriate blood oxygen saturations. It is unclear whether co-morbidities increase disease
190 by changing spatial and temporal expression of SARS-CoV-2 receptors or the immune response that
191 leads to a lethal cytokine storm¹. In this study, we present evidence that expression of the SARS-
192 CoV-2 co-receptors ACE2 and TMPRSS2 increase in the respiratory epithelium of mice as they age
193 and this can be stimulated or accelerated by early exposure to hyperoxia. Expression of ACE2 in
194 distal AT2 cells was of particular interest because infection of these cells with other viruses has been
195 associated with higher mortality in humans¹⁵⁻¹⁷. When infected such as by influenza A virus, AT2 cells
196 may contribute to lung disease by producing inflammatory molecules that contribute to a lethal
197 cytokine storm¹². They may also die and therefore reduce the number of surviving AT2 cells required
198 to serve as stem cells for alveolar regeneration^{22,29,30}. Our findings support the idea that age and co-
199 morbidities like preterm birth may increase the severity of COVID-19 by changing temporal and spatial
200 patterns of SARS-CoV-2 receptors.

201 We found that ACE2 was primarily expressed by airway Club cells during early postnatal life.
202 The intensity of ACE2 staining increased in the airways of mice with age and became detectable in
203 the alveoli of young adult mice. Co-localization with proSP-C revealed that most, but not all alveolar
204 cells expressing ACE2 were AT2 cells. Our findings are consistent with an earlier study showing that
205 ACE2 is expressed in the adult mouse lung by Clara cells (now called Club cells), AT2 cells, and to
206 some extent by endothelial cells around small and medium sized vessels³¹. While that study showed
207 how ACE2 levels rise during fetal development, our findings extend it by showing that ACE2

208 expression continues to increase as mice age. We also found that *Tmprss2* mRNA expression
209 increases as mice age and this expression was similarly enhanced by neonatal hyperoxia. While AT2
210 cells have previously been shown to express TMPRSS2¹¹, we were not able to detect it in the mouse
211 lung using commercially available antibodies. However, we did find that the abundance of *Tmprss2*
212 mRNA and protein abundance increased with age and neonatal hyperoxia, and was reduced by
213 mitoTEMPO similar to that of *Ace2*. The higher expression of these genes as mice age is in
214 agreement with recent review that discussed two unpublished studies deposited in *bioRxiv* showing
215 how expression of *Ace2* and *Tmprss2* mRNA increases with age in human respiratory epithelium¹.
216 Those findings in humans and ours in mice suggest the age-dependent increase in SARS2-CoV-2
217 receptors may be responsible for increasing the severity of COVID-19 lung disease in elderly people.

218 It is important to recognize the normal functions of ACE2 and TMPRSS2 because that may
219 help explain why their expression steadily increases with age³². ACE2 is perhaps best known for its
220 role in controlling blood pressure in the renin-angiotensin system³³. ACE1 converts the 10-amino acid
221 angiotensin I to an 8-amino acid vasoconstrictive peptide called angiotensin II. ACE2 accumulates in
222 people with pulmonary hypertension and hydrolyzes Angiotensin II to Ang(1-7), which has
223 vasodilation properties. Over-expressing ACE2 also protects against right ventricular hypertrophy³⁴.
224 Hence, higher levels of ACE2 seen as the lung ages may reflect an adaptive response designed to
225 protect against the development of cardiovascular disease. Interestingly, ACE2 levels decline in
226 bleomycin-induced lung fibrosis and humans with interstitial pulmonary fibrosis while angiotensin II
227 levels rise^{35,36}. Angiotensin II can promote fibrosis by stimulating AT2 cell apoptosis downstream of
228 TGF- β signaling³⁷. ACE2 serves as an anti-fibrotic molecule by stimulating the hydrolysis of
229 angiotensin II to Ang(1-7), which in turn signals through the Mas oncogene to block AT2 cell apoptosis
230 by suppressing JNK activation³⁸. The slow and steady increase in ACE2 expression as the lung ages
231 may also serve to preserve AT2 cells and thus reduce or prevent the development of idiopathic
232 pulmonary fibrosis. In contrast to ACE2, the normal role of TMPRSS2 in the lung is poorly understood.
233 TMPRSS2 is a serine protease that is localized to the apical surface of secretory cells such as Club
234 and AT2 cells of the lung³⁹. Its expression is highly regulated by androgens in the prostate gland and

235 may be similarly responsive to androgens in the lung, suggesting it may play a role in sex-dependent
236 differences in the lung.

237 Our study also found that neonatal hyperoxia increased or accelerated expression of *Ace2*
238 mRNA, ACE2 protein, and *Tmprss2* mRNA as mice age. Significant changes were seen with 60% or
239 more FiO₂ at 8 weeks (2 months) of age and persisted as mice age. How hyperoxia regulates
240 expression of these proteins is conflicting and remains to be better understood. One study using
241 human fetal IMR-90 fibroblasts found that hyperoxia does not change expression of ACE2⁴⁰.
242 However, ACE2 was depleted when cells returned to room air presumably because it was being
243 proteolyzed and shed into the media. In contrast, another study found higher levels of ACE2 in
244 newborn rats exposed to 95% oxygen for the first week of life and then recovered in 60% oxygen for
245 the next two weeks⁴¹. In our hands, changes in *Ace2* or *Tmprss2* mRNA were first detected in 8-
246 week-old mice exposed to hyperoxia between PND0-4. We did not detect changes at the end of
247 oxygen exposure (PND4). In fact, we recently deposited an RNA-seq analysis of AT2 cells isolated
248 from PND4 mice exposed to room air versus hyperoxia that shows hyperoxia modestly inhibits *Ace2*
249 and increases *Tmprss2* mRNA abundance (Gene Expression Omnibus of the National Center for
250 Biotechnology Information under the accession number GSE140915). This suggests neonatal
251 hyperoxia may not affect expression until after the mice are returned to room air. Because ACE2 and
252 TMPRSS2 were only affected by doses of oxygen that cause long-term changes in lung function (i.e.,
253 60% for 4 days but not 40% for 4 or 8 days), we speculate that they occur as an adaptive response to
254 the alveolar simplification and cardiovascular disease as mice exposed to neonatal oxygen age. The
255 elevated expression of ACE2 and perhaps TMPRSS2 may serve to prevent the loss of AT2 cells
256 damaged by early oxygen and promote vasodilation as the pulmonary capillary bed undergoes
257 rarefaction^{23,42}. But higher levels of these proteins may become a maladaptive response when they
258 render the lung more susceptible to coronavirus infections.

259 While it remains to be determined how age or oxygen regulate expression of ACE2 and
260 TMPRSS2, our studies with mitoTEMPO suggest their expression may be influenced by oxidative
261 stress. Administering mitoTEMPO, a scavenger of mitochondrial superoxide during hyperoxia blunted

262 the oxygen-dependent increase in these genes detected in 2-month-old mice. Because hyperoxia
263 progressively increases mitochondrial oxidation, it has historically been used to model aging-related
264 oxidative stress⁴³. This implies mitochondrial oxidation that accumulates as the lung ages steadily
265 increases expression of ACE2 and TMPRSS2, which in turn may then attempt to defend against the
266 pathological changes attributed to the aging process. Anti-oxidant therapies may therefore prove
267 useful for suppressing expression of SARS-CoV-2 receptors and reducing the severity of COVID-19
268 related lung disease, especially in people with pre-existing co-morbidities.

269 Increased expression of ACE2 and TMPRSS2 may not be the only way these proteins
270 enhance the severity of COVID-19-related lung disease. For example, TMPRSS2 facilitates viral
271 activation and entry by cleaving hemagglutinin on influenza A virus and the spike protein on the
272 SARS-CoV-2 virus¹¹. The spike protein accesses the cell when it binds the glucose regulated protein
273 78 (Grp78, BiP) found on the cell surface⁴⁴. Grp78 is a master regulator of the unfolded protein
274 response (UPR)⁴⁵. It is normally localized to the endoplasmic reticulum (ER) where it inhibits the UPR
275 by binding Activating Transcription Factor 6 (ATF6), Protein kinase RNA-like Endoplasmic Reticulum
276 Kinase (PERK), and Inositol-requiring Enzyme 1 (IRE1). Grp78 is released from these proteins when
277 oxidation and other stressful conditions cause an accumulation of unfolded proteins. It can then
278 escape the ER and traffic to the cell surface where it becomes available to bind the coronavirus S
279 protein and facilitate viral entry. This information should raise great concern for people with familial
280 forms of IPF caused by mutations in *SFTPC* and other genes that activate the UPR in AT2 cells⁴⁶.
281 Genetic studies in mice suggest mutant forms of SP-C that activate the UPR are not sufficient by
282 themselves to cause fibrotic lung disease. However, they can predispose the lung to fibrotic disease
283 following viral infections⁴⁷. Familial forms of IPF that activate the UPR in AT2 cells may therefore
284 accelerate the age-dependent susceptibility of AT2 cells to SARS-CoV-2 infections.

285 In summary, we found that neonatal hyperoxia increases or accelerates the age-dependent
286 expression of ACE2 and TMPRSS2 in the airway and alveolar epithelium of mice. Understanding how
287 expression of these proteins changes with age and in response to early life insults such as neonatal

288 hyperoxia may provide new opportunities for reducing the severity of COVID-19 and other types of
289 lung disease.

290 **MATERIALS AND METHODS**

291 *Mice.* C57BL/6J mice were purchased from the Jackson Laboratories and maintained as an
292 inbred colony. Mice were exposed to room air (21% oxygen) as control or hyperoxia (100% oxygen
293 unless otherwise stated) between birth and postnatal day (PND) 4 and then returned to room air¹⁹.
294 Dams were cycled every 24 hours to ensure that hyperoxia did not compromise their health. Some
295 mice exposed to room air or hyperoxia were injected intraperitoneally with 0.7µg/g mitoTEMPO (Enzo
296 Life Sciences, Farmingdale, NY) or vehicle (phosphate-buffered saline) on PND0, PND1, and PND2.
297 All mice used in this study were of mixed sex and housed in a pathogen-free environment according
298 to a protocol (UCAR2007-121E) approved by the University Committee on Animal Resources at the
299 University of Rochester.

300

301 *Immunohistochemistry.* Lungs were inflation fixed overnight in 10% neutral buffered formalin,
302 embedded in paraffin and 4 µm sections prepared^{23,48}. Sections were stained with antibodies against
303 ACE2 (Invitrogen, PA5-47488, Waltham, MA), Scgb1a1 (Sigma, 07-063, St. Louis, MO) and proSP-C
304 (Seven Hills Bioreagents, Cincinnati, OH). Immune complexes were detected with fluorescently
305 labeled secondary antibody (Jackson Immune Research, West Grove, PA). Sections were then
306 stained with 4', 6-diamidino-2-phenylindole (DAPI) (Life Technologies, Carlsbad, CA) before viewing
307 with Nikon E800 Fluorescence microscope (Microvideo Instruments, Avon, MA) and a SPOT-RT
308 digital camera (Diagnostic Instruments, Sterling Heights, MI).

309

310 *Quantitative RT-PCR.* Total RNA was isolated from the lung using Trizol reagent
311 (ThermoFisher Scientific) and reverse transcribed to cDNA using the iScript cDNA synthesis kit (Bio-
312 Rad Laboratories, Hercules, CA). The cDNA was then amplified with SYBR Green I dye on CFX96™
313 or CFX384™ Real-Time PCR detection system (Bio-Rad Laboratories, Hercules, CA). PCR products
314 were amplified with sequence-specific primers for mouse *Ace2* (sense 5'-
315 GGATACCTACCCTTCCTACATCAGC-3' and antisense CTACCCACATATCACCAAGCA-3'),
316 *Tmprss2* (sense 5'- TACTTGGAGCGGACGAGGAA-3', and antisense 5'-

317 AGGAGGTCAGTATGGGGCTT-3') or 18S rRNA (sense 5-CGGCTACACATCCAAGGAA-3', and
318 antisense 5'- GCTGGAATTACCGCGGCT- 3') used to normalize equal loading of the template
319 cDNAs. Amplifications were conducted with iTaq Universal SYBR Green Master Mix (Bio-Rad
320 Laboratories, Hercules, CA). Fold changes in gene expression were calculated by the $\Delta\Delta C_t$ method
321 using the C_t values for the housekeeping 18S rRNA as a control for loading.

322

323 *Western blot analysis.* The left lung lobe was homogenized in lysis buffer and insoluble
324 material removed by centrifugation²³. Equal amounts of protein were separated on sodium dodecyl
325 sulfate polyacrylamide gels and transferred to nylon membranes. The membranes were
326 immunoblotted with primary antibodies to ACE2 (Invitrogen, PA5-47488, Waltham, MA), TMPRSS2
327 (Abcam, ab92323, Cambridge, MA) or β -ACTIN (Sigma, A2066). The blots were then incubated in
328 appropriate secondary antibody (Southern Biotech, Birmingham, AL). Immune complexes were
329 detected by chemiluminescence and visualized with a ChemiDoc Imaging System (Bio-Rad
330 Laboratories, Hercules, CA).

331

332 *Statistical Analysis.* Data were evaluated using JMP14 software (SAS Institute, Cary, NC) and
333 graphed as means \pm SEM. An unpaired t-test and 2-way ANOVA were used to determine overall
334 significance, followed by Tukey-Kramer HSD tests.

335

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471

472 **AUTHOR CONTRIBUTIONS**

473 M.Y. designed and conducted experiments, analyzed the data, prepared figures, and helped
474 write the manuscript; E.C. performed experiments and helped write the manuscript; J.H., performed
475 experiments; A.D. aided in experimental design; M.O. designed the experimental research, analyzed
476 the data, and wrote the manuscript. All authors reviewed and approved the final version of the
477 manuscript.

478

479 **COMPETING INTEREST STATEMENT**

480 The author(s) declare no competing interests.

481 **FIGURE LEGENDS**

482

483 **Figure 1.** ACE2 expression changes in lung as mice age. **(a)** Lungs harvested from mice of different
484 ages were stained for ACE2 (red) and counterstained with DAPI (blue). ACE2 was detected in
485 airways of all mice and alveolar regions (yellow arrows). Bar = 100 μ m. **(b)** Lungs homogenates
486 prepared from 2-month, 12-month, and 24-month-old mice were immunoblotted for ACE2 and β -
487 ACTIN as a loading control. Each lane represents an individual mouse. Band intensity of ACE2 to β -
488 ACTIN was quantified and graphed as fold change relative to 2-month samples. Bars reflect mean \pm
489 SD graphed. **(c)** qRT-PCR was used to quantify *Ace2* mRNA in total lung homogenates of 2-month
490 and 24-month-old mice. Data is graphed as the fold change of *Ace2* after normalizing to *18S* RNA.
491 Bars reflect mean \pm SD graphed as fold change over 2-month values. Statistical significance is
492 comparisons for all pairs using Tukey-Kramer HSD test, with * $P \leq 0.05$; ** $P \leq 0.01$.

493

494 **Figure 2.** Aging increases ACE2 expression in airway Club and alveolar type 2 cells. **(a)** Lungs from
495 2-month and 12-month-old mice were immunostained for ACE2 (red), *Scgb1a1* (green), and
496 counterstained with DAPI (blue). Boxed sections are individual ACE2 and *Scgb1a1* stains. **(b)**
497 Quantitation of ACE2 Red staining intensity. All the cells were imaged using identical exposure time.
498 Scale bar = 50 μ m. **(c)** Lungs were stained for ACE2 (red), proSP-C (green), and counterstained with
499 DAPI (blue). Boxed sections are enlarged below each figure. **(d)** The proportion of proSP-C+ cells
500 expressing ACE2 was quantified and graphed. Statistical significance is comparisons for all pairs
501 using Tukey-Kramer HSD test, with ** $P \leq 0.01$; *** $P \leq 0.001$. Bar = 50 μ m.

502

503 **Figure 3.** Neonatal hyperoxia stimulates expression of ACE2 in adult mice. **(a)** Cartoon showing the
504 experimental approach of exposing newborn mice to hyperoxia. **(b)** Total lung homogenates were
505 immunoblotted for ACE2 and β -ACTIN as a loading control. Data is graphed as mean \pm SD fold
506 change over room air values. **(c)** qRT-PCR was used to quantify *Ace2* mRNA in total lung

507 homogenates of 2-, 12-, and 18-month-old mice exposed to room air or hyperoxia between PND0-4.
508 Values were normalized to expression of 18S RNA and graphed as mean \pm SD fold change of ACE2
509 in 2-month-old room air mice. **(d)** qRT-PCR was used to quantify *Ace2* mRNA in total lung
510 homogenates of 2-month-old mice exposed to room air, 40%, 60%, or 80% oxygen between PND0-4.
511 Values were normalized to expression of 18S RNA and graphed as fold change of ACE2 in 2 month
512 room air mice. Statistical significance is comparisons for all pairs using Tukey-Kramer HSD test with
513 * $P \leq 0.05$; ** $P \leq 0.01$; *** $P \leq 0.001$.

514

515 **Figure 4.** Neonatal hyperoxia stimulates expression of ACE2 in alveolar type 2 cells. **(a)** Lungs of 2-,
516 6- and 12-month-old mice exposed to room air or hyperoxia between pnd0-4 were stained for ACE2
517 (red), proSP-C (green), and DAPI. Upper rows reflect room air and lower rows reflect hyperoxia
518 between PND0-4. Boxed regions are enlarged to the right of each image. **(b)** The proportion of ACE2-
519 positive to total DAPI cells was quantified and graphed. **(c)** The proportion of proSP-C+ cells that
520 express ACE2 were quantified and graphed. Values in b, c represent mean \pm SD of 4-5 lungs per
521 group with stated P values in the graphs. Statistical significance is comparisons for all pairs using
522 Tukey-Kramer HSD test with * $P \leq 0.05$.

523

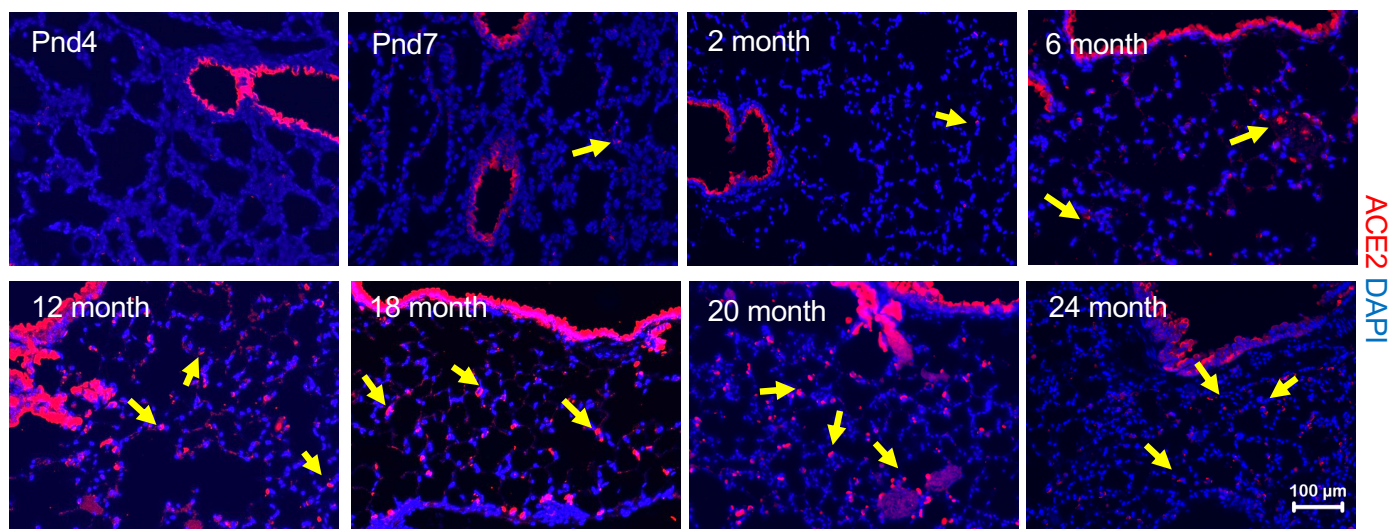
524 **Figure 5.** Anti-oxidants prevent hyperoxia from stimulating expression of ACE2. **(a)** Cartoon showing
525 the experimental approach of exposing newborn mice to hyperoxia and treated with mitoTEMPO (d1-
526 d3). **(b)** qRT-PCR was used to measure *Ace2* mRNA expression in 2-month-old mice exposed to
527 room air or hyperoxia as vehicle or mitoTEMPO between PND0-4. Values reflect mean \pm SD of 4-5
528 mice per group and graphed as fold change over mice administered room air and vehicle control.
529 Expression of *Ace2* mRNA was normalized to 18S rRNA and mean \pm SD values graphed relative to
530 room air values. **(c)** Lung alveoli were stained for ACE2 (red), and counterstained with DAPI (blue).
531 **(d)** Total % of ACE2 cells in lung alveoli. **(e)** Lung-airways were stained for ACE2 (red), and
532 counterstained with DAPI (blue). **(f)** Quantitation of ACE2 Red staining intensity. All the cells were
533 imaged using identical exposure time. Scale bar = 50 μm ; Quantitation of ACE2 Red was derived from

534 images. Statistical significance is comparisons for all pairs using Tukey-Kramer HSD test with
535 *P≤0.05; **P≤0.01; ***P≤0.001.

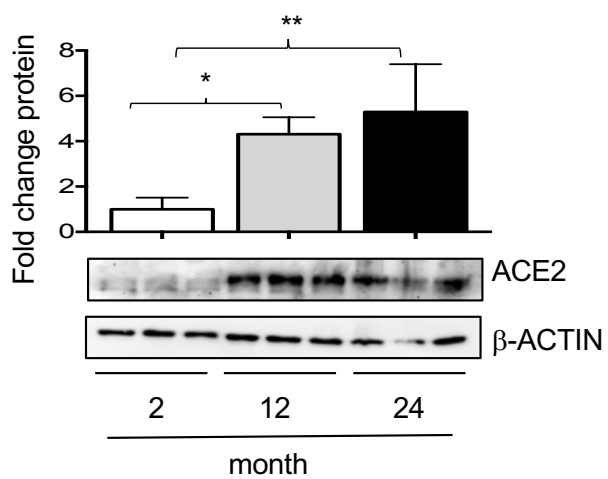
536

537 **Figure 6.** Neonatal hyperoxia stimulates age-dependent expression of *Tmprss2* mRNA. **(a)** qRT-PCR
538 was used to quantify *Tmprss2* mRNA in total lung homogenates of 2-, 12-, and 18-month-old mice
539 exposed to room air or hyperoxia between PND0-4. Values were normalized to expression of *18S*
540 RNA and graphed as fold change of ACE2 in 2-month-old room air mice. **(b)** Western blot-based
541 quantification of TMPRSS2. Data in panels A-D reflect mean ± SD and graphed as fold change
542 relative to control mice exposed to room air. **(c)** qRT-PCR was used to measure *Tmprss2* mRNA in
543 total lung homogenates of 2 month mice exposed to room air, 40%, 60%, or 80% oxygen between
544 PND0-4. **(d)** qRT-PCR was used to measure *Tmprss2* mRNA in control and 2-month-old mice
545 exposed to room air or hyperoxia and vehicle or mitoTEMPO between PND0-4 N=4-5 mice per group.
546 Statistical significance is comparisons for all pairs using Tukey-Kramer HSD test with *P≤0.05;
547 **P≤0.01.

a



b



c

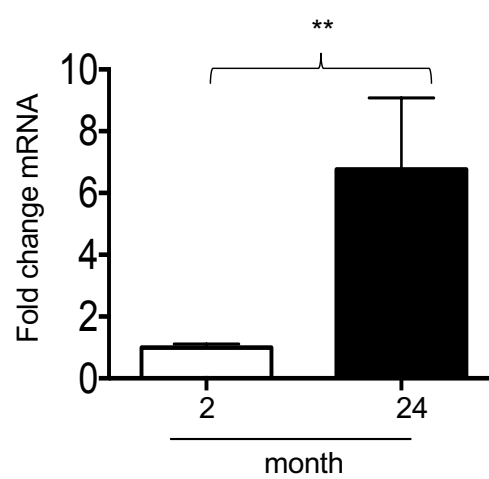
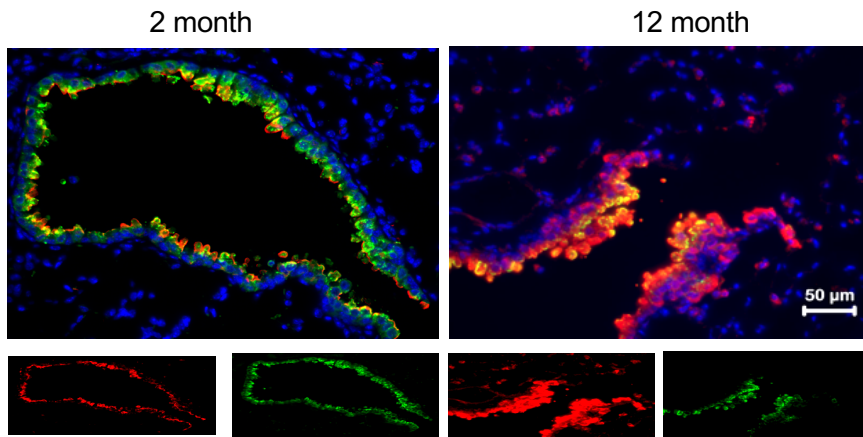
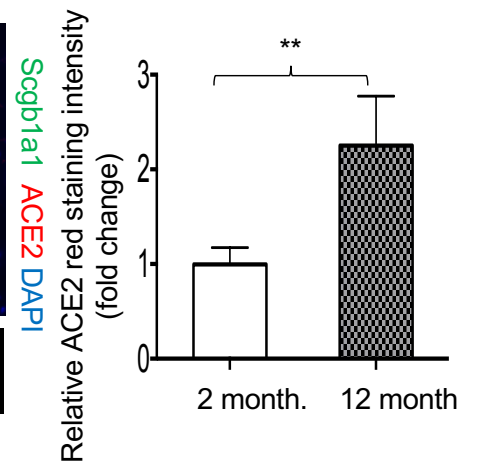


Figure 1

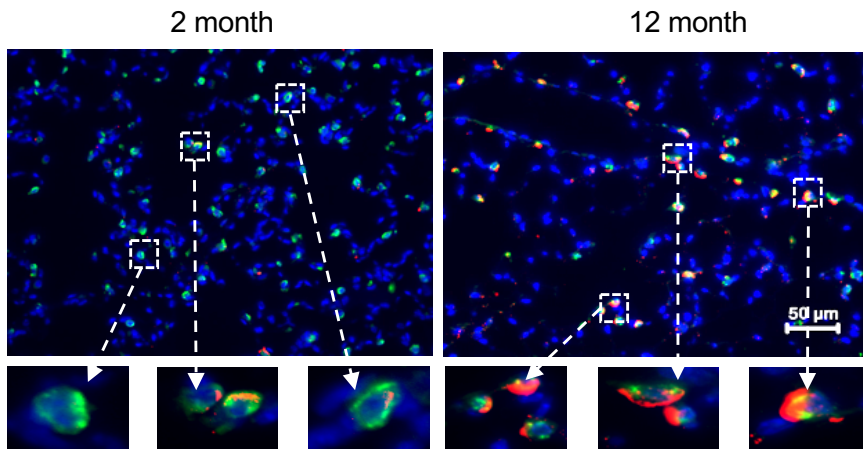
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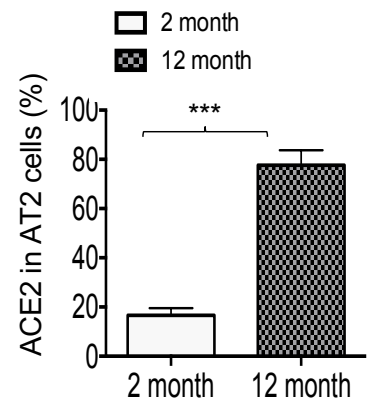


Figure 2

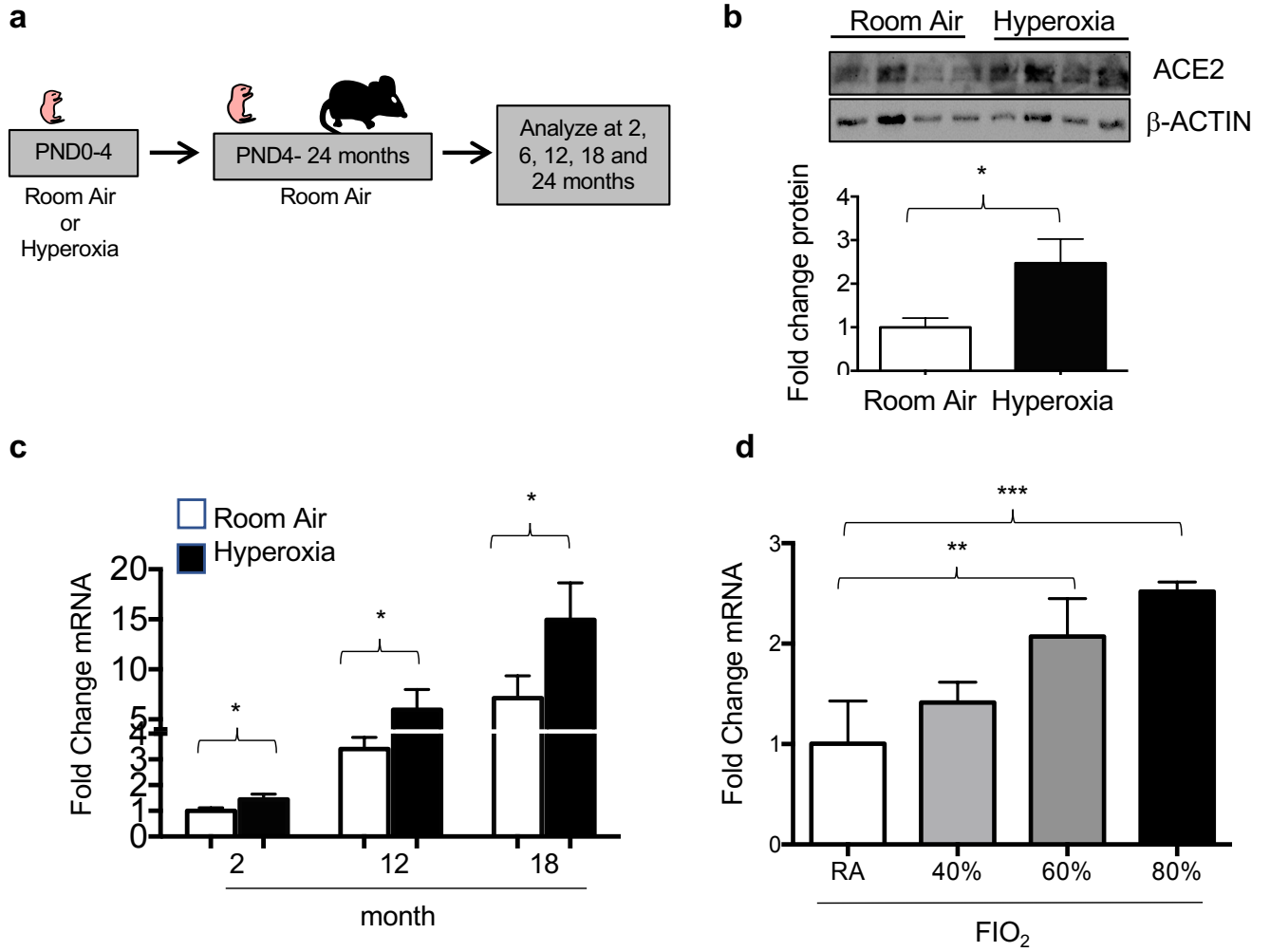


Figure 3

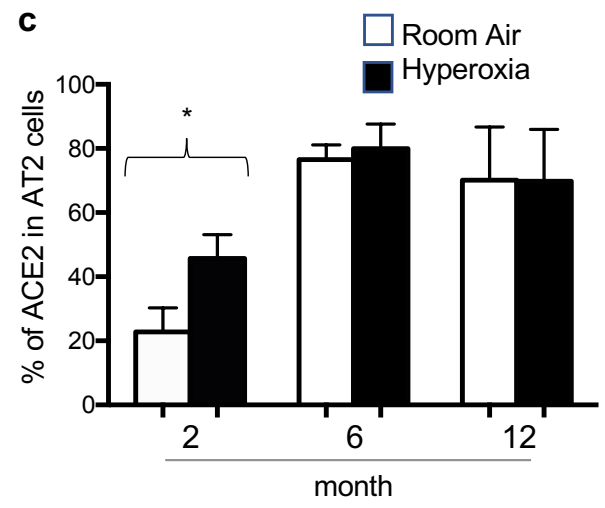
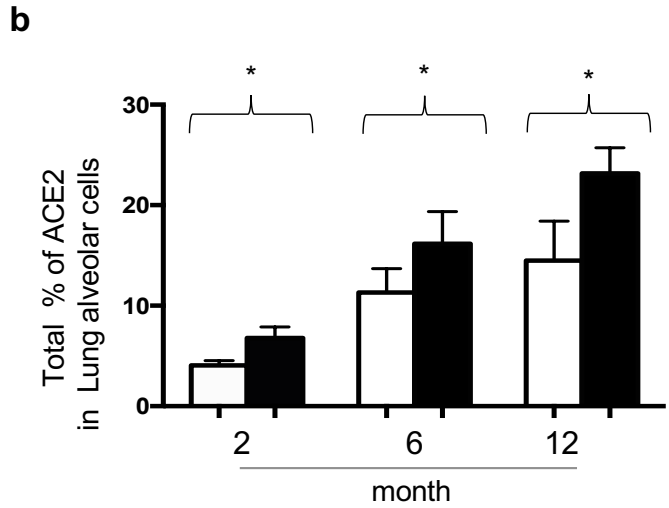
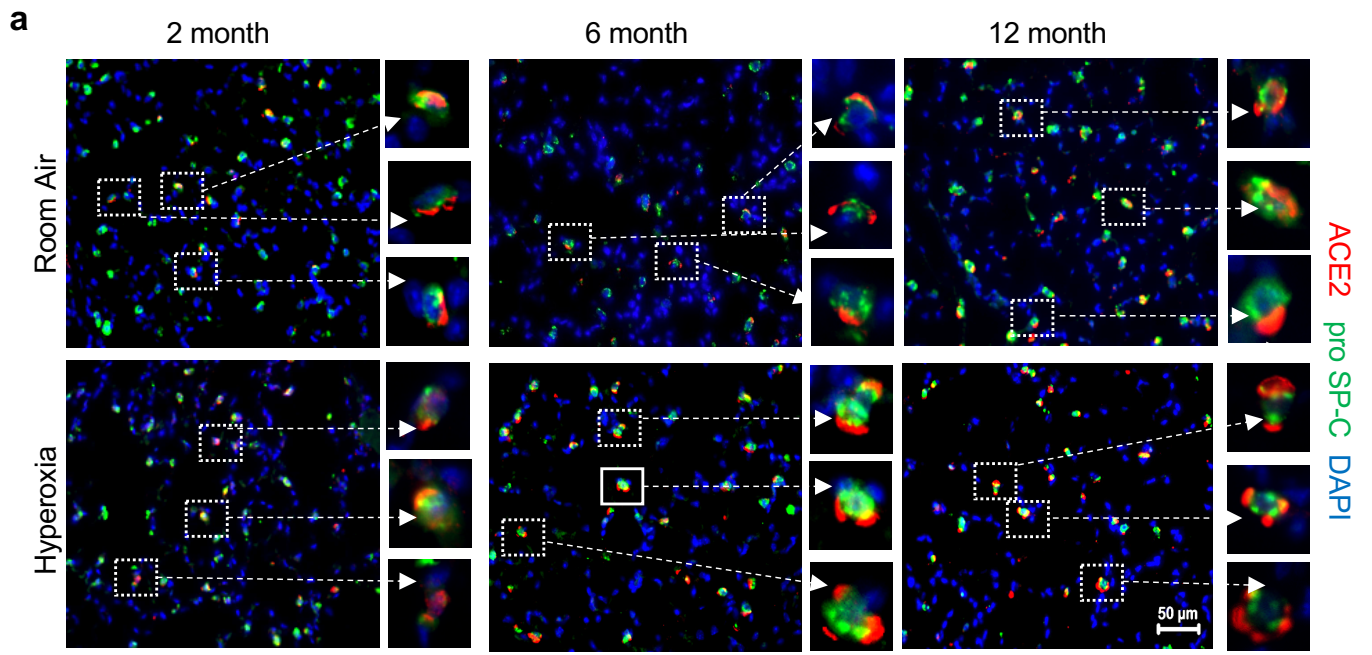


Figure 4

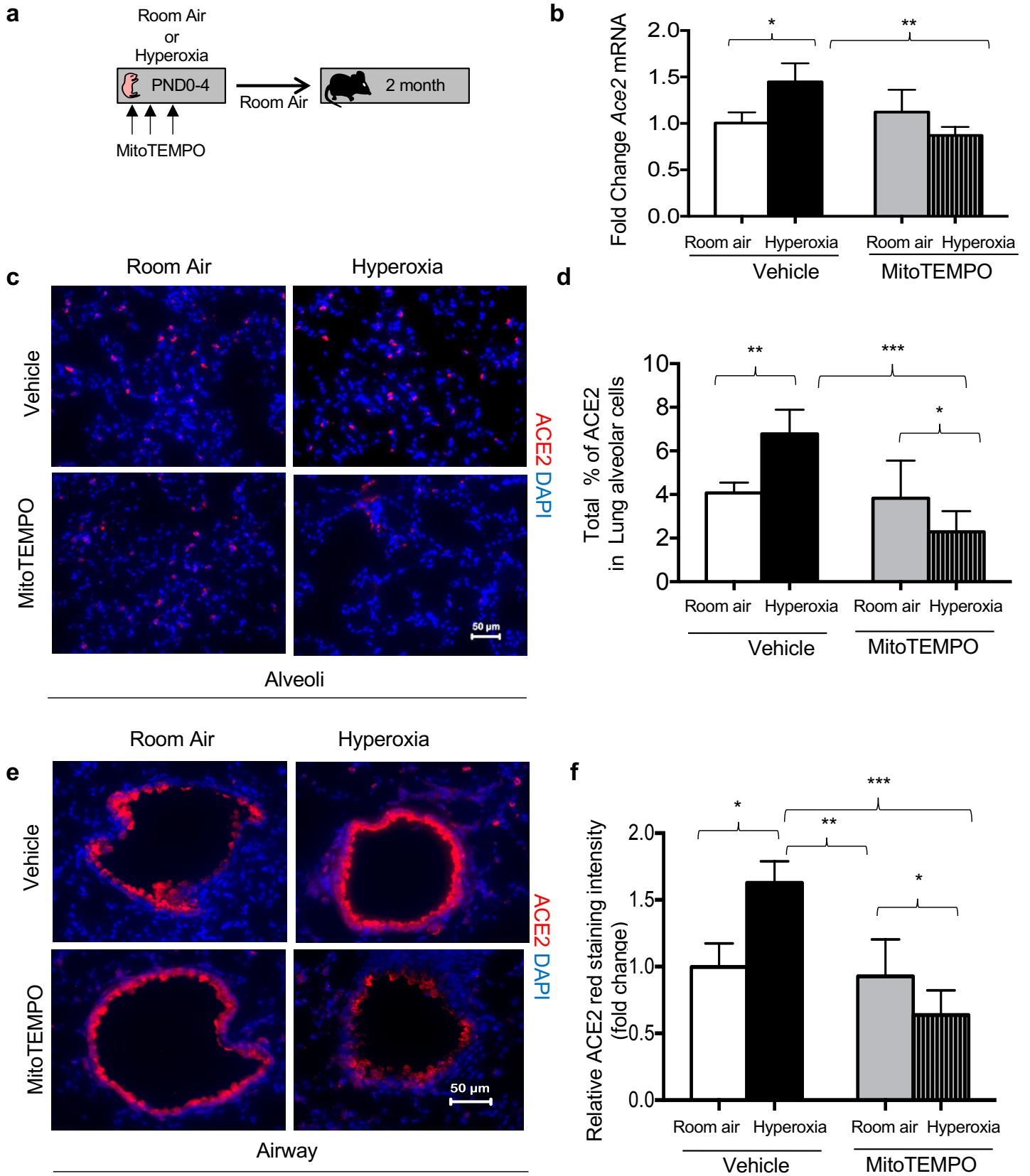


Figure 5

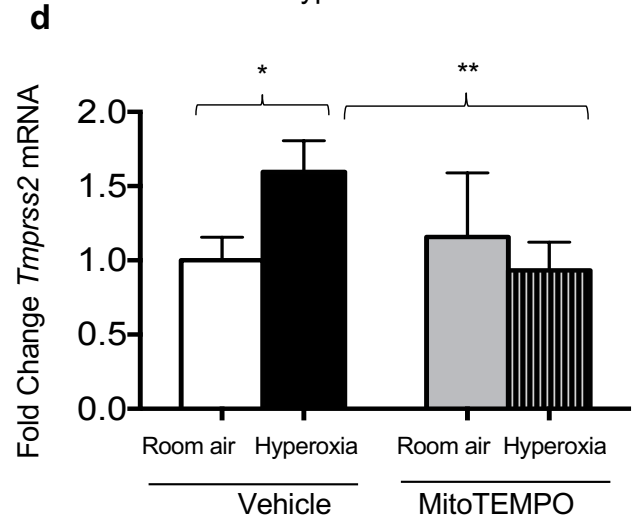
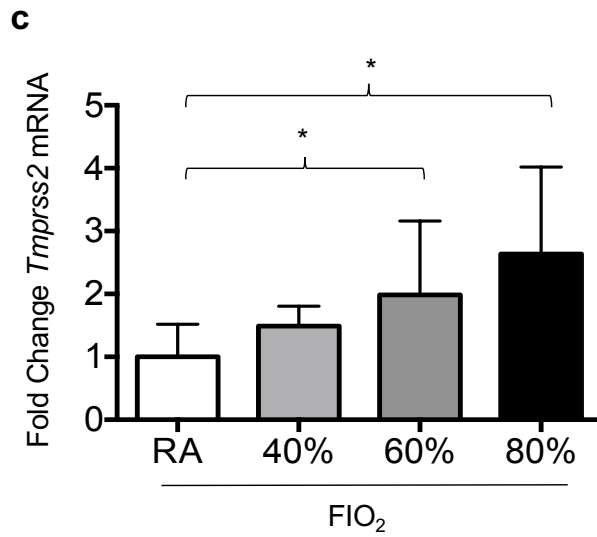
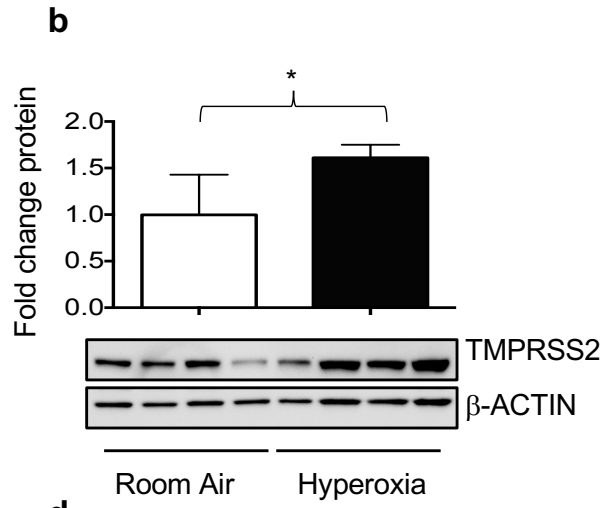
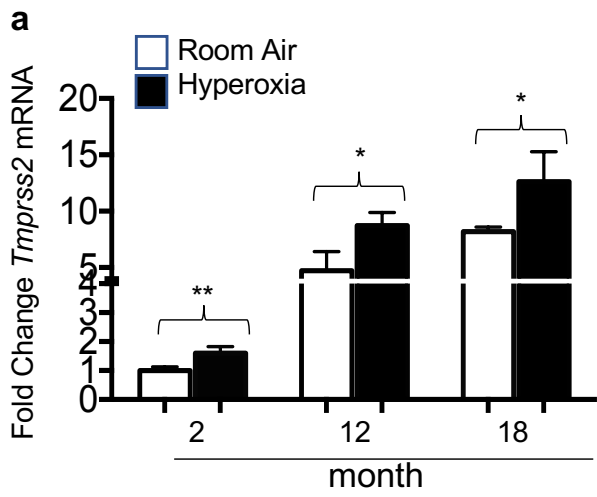


Figure 6